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Cloning, Sequencing and Regulation of the Rhodobacter Capsulatus hemH Gene.

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CLONING, SEQUENCING AND REGULATION OF THE *Rhodobacter capsulatus hemH* GENE

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ABSTRACT

Rhodobacter capsulatus, a purple non-sulfur photosynthetic bacterium, synthesizes four tetrapyrrole end products: heme, bacteriochlorophyll, siroheme and vitamin B_{12} via the common tetrapyrrole biosynthetic pathway. The biosynthesis of tetrapyrroles in *R. capsulatus* is a complex and precisely controlled process which has been under extensive investigation.

In order to study the regulation of tetrapyrrole biosynthesis, the *R. capsulatus hemH* gene was cloned by complementation of an *E. coli* Δ*hemH* mutant and sequenced. The enzyme ferrochelatase, encoded by the *hemH* gene, catalyzes the insertion of ferrous iron into protoporphyrin IX, thus producing heme. The consensus ferrochelatase signature sequence was found in the *R. capsulatus hemH* gene sequence, with the exception of one amino acid substitution.

Mapping of the *hemH* gene to the *R. capsulatus* chromosome confirmed that the *hem* genes located so far are not arranged in a cluster.

Overexpression of the *hemH* gene in *R. capsulatus* was used to study the effect of excess heme on the regulation of tetrapyrrole biosynthesis. The overexpression resulted in increased ferrochelatase activity, decreased aminolevulinate synthase activity and reduced levels of porphyrins and bacteriochlorophyll. This was the first evidence for feedback inhibition of

aminolevulinate synthase by heme in *R. capsulatus*. These *in vivo* results were also confirmed by *in vitro* experiments.

In order to determine if oxygen and heme participate in a single regulatory mechanism, a coproporphyrin-accumulating *R. capsulatus* mutant was grown in the presence of execs heme under high and low oxygen tension. The results clearly indicate that regulation of tetrapyrrole biosynthesis by heme is separate from regulation by oxygen.

INTRODUCTION

Purple, nonsulfur photosynthetic bacteria are a remarkably diverse group of organisms with regard to their mechanisms of obtaining energy. *Rhodobacter capsulatus*, a particularly interesting member of that group, is capable of using a number of growth modes. It can grow anaerobically either as a photoautotroph on H_2 and CO_2 or as a photoheterotroph on a variety of organic carbon sources (137). In addition, *R. capsulatus* can grow as a fermentative anaerobe in darkness with sugars as sole energy and carbon sources, provided that dimethyl sulfoxide or trimethylamine-N-oxide is added (136,138). *R. capsulatus* can also achieve aerobic growth as a chemoheterotroph in darkness or as a chemoautotroph in darkness with H_2 as the electron source (137). The ability of *R. capsulatus* to grow in at least five distinct growth modes makes it one of the most metabolically versatile organisms. Another feature that contributes to the uniqueness of this organism is its ability to synthesize all four tetrapyrrole endproducts: bacteriochlorophyll, heme, vitamin B_{12} and siroheme, via the common tetrapyrrole pathway. Bacteriochlorophyll is the main light-harvesting pigment. Heme serves as a prosthetic group in cytochromes, catalases and peroxidases. Vitamin B_{12} serves as a cofactor of the homocysteine methyltranferase which is involved in methionine biosynthesis (35). Siroheme functions as a prosthetic group for the sulfite reductase (149) and nitrite reductase (151). All four tetrapyrrole end products are functionally very

different and are produced in dramatically different amounts depending on the growth conditions and other requirements. For example, in photosynthetically grown cultures of *R. capsulatus*, bacteriochlorophyll production could reach up to 98% of the total tetrapyrrole end product. Conversely, aerobically grown cultures produce no bacteriochlorophyll. This suggests the existence of a very complex and precise regulatory mechanism to control the amounts of tetrapyrrole end products synthesized via the tetrapyrrole pathway in response to growth conditions and demand.

Tetrapyrrole biosynthesis

The tetrapyrroles comprise a diverse group of natural compounds that share a common, multiply-branched biosynthetic pathway (Fig. 1). The common portion of the pathway which includes the biosynthetic steps from aminolevulinate to protoporphyrin IX is similar or identical in all living systems studied so far. There are only few types of organisms with incomplete tetrapyrrole pathways (77). It has been determined, that in order to grow they require some exogenous source of tetrapyrrole such as heme or a tetrapyrrole precursor. For example, *Haemophilus influenzae* can grow only if the medium is supplemented with an exogenous tetrapyrrole. It has been established that protoporphyrin IX can be added to the medium instead of heme, suggesting that the organism can insert iron into the protoporphyrin IX ring, thus producing heme (76). There have been no examples of organisms that neither make nor require tetrapyrroles described so far.

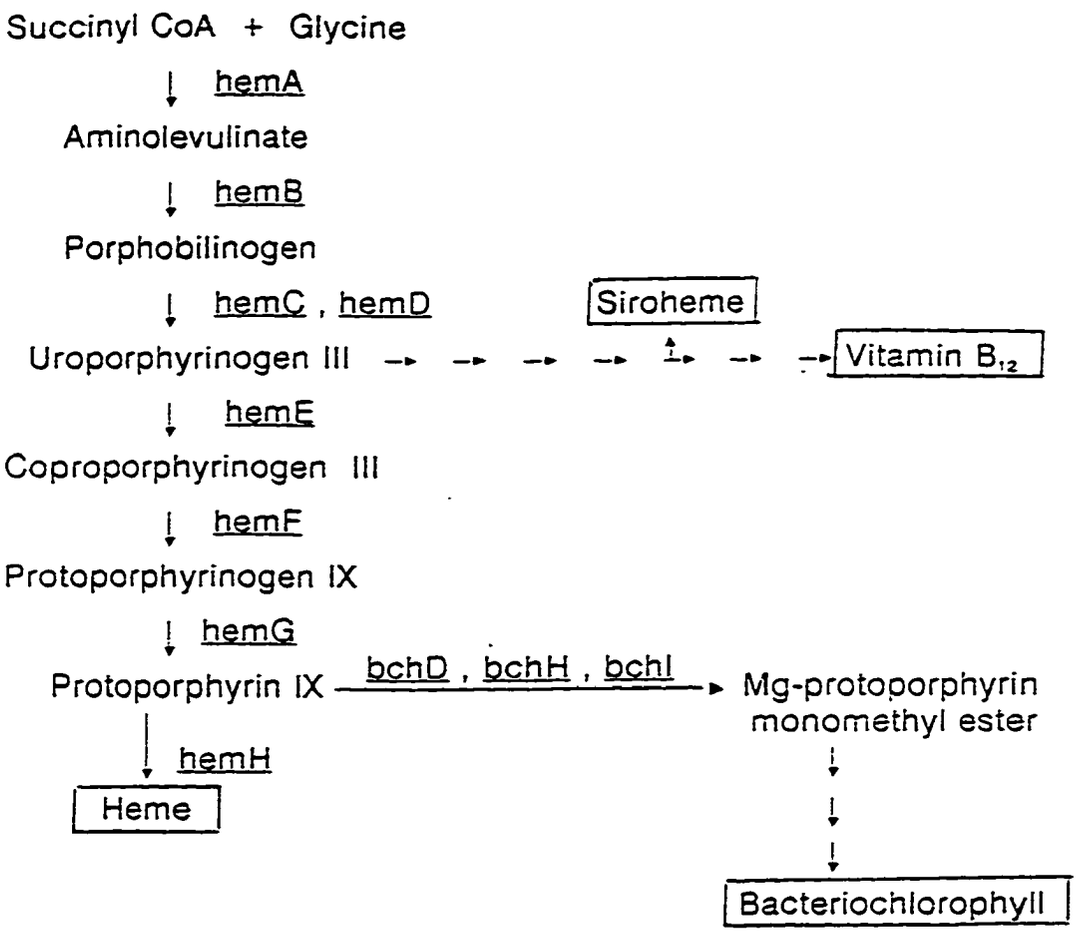


Figure 1. The tetrapyrrole biosynthetic pathway in *R. capsulatus*.

The first biochemical intermediate in the tetrapyrrole biosynthetic pathway is aminolevulinate, a five-carbon aminoketone. Its formation was first demonstrated in extracts of the photosynthetic bacterium *Rhodobacter spaeroides* (119) and avian erythrocytes (72). Two distinct mechanisms exist by which aminolevulinate is produced. One of these mechanisms involves condensation of glycine with succinyl CoA, a reaction catalyzed by the pyridoxal phosphate-requiring enzyme aminolevulinate synthase (119). This route of aminolevulinate formation is characteristic for animals (66), yeast and fungi (66), and some bacterial species such as the purple nonsulfur photosynthetic bacteria *R. capsulatus* (7). The aminolevulinate synthase enzyme is the product of the *hemA* gene. The enzyme has been purified from variety of sources including *Saccharomyces cerevisiae* (205), *Paracoccus denitrificans* (193), chicken and rat liver (176). It has not yet been purified from *R. capsulatus*, but there are reports of its purification from another *Rhodobacter* species, *R. sphaeroides* (108,206). All of the purified aminolevulinate synthase enzymes exist as homodimers with subunit molecular masses between 40,000 and 70,000 Da (108,159,205). Enzymatic activity has been shown to be activated by trisulfides (170) and inhibited by heme (29) and magnesium protoporphyrin (221). Genes encoding aminolevulinate synthases have been cloned and characterized in several bacteria (60,133,142). The *R. capsulatus hemA* gene has been cloned independently by Biel et al. (22) and Hornberger et al. (91). Surprisingly, in *R.*

sphaeroides, a bacterium related to *R. capsulatus*, two genes (*hemA* and *hemT*) are responsible for aminolevulinate synthesis (191). The *hemA* and *hemT* gene sequences were found to be 65% identical to each other (157). The second mechanism of aminolevulinate synthesis is found in plants (12), algae (209) and most bacterial groups (6,63,69,89,143), suggesting that it has probably evolved earlier in evolution. Aminolevulinate is formed from the five-carbon skeleton of glutamate, therefore, the route of its formation is called the five-carbon pathway. The widely accepted model for aminolevulinate synthesis via the five-carbon pathway consists of three enzymatic steps. In the first one, glutamate is activated by an aminoacyl-tRNA synthetase, thus forming glutamyl-$tRNA^{Glu}$ (115,177). This is a reaction resembling the tRNA activation reaction in protein biosynthesis and it requires ATP and Mg^{2+}. In the next step, the carboxyl group of the tRNA-bound glutamate is reduced by an NADPH-dependent reductase, thus forming glutamate 1-semialdehyde (90). Finally, the glutamate 1-semialdehyde is converted to aminolevulinate via a transamination reaction catalyzed by a glutamate 1-semialdehyde aminotransferase (63,209).

The second step in the tetrapyrrole pathway is the synthesis of the first pyrrole in the pathway, porphobilinogen. It is formed by condensation of two aminolevulinate molecules with elimination of two molecules of water, a reaction catalyzed by the enzyme porphobilinogen synthase (5-aminolevulinic acid dehydratase) (77). This enzyme has been found virtually in all living

systems and has been purified and studied in a variety of sources such as plants (139,180), human erythrocytes (4), bovine liver (213), and bacteria (88, 217). The porphobilinogen synthase has an octameric structure and is composed of identical subunits with native molecular masses in the range of 25,000 to 35,000 Da (111,155). The porphobilinogen synthase enzymes from different sources vary according to their metal requirements. In mammals, yeast and some bacteria the enzyme requires zinc ions for activity (111). The enzyme present in plants requires magnesium ions for maximal activity (181), whereas the *R. sphaeroides* enzyme requires monovalent cations such as potassium ions (155). The *R. capsulatus* porphobilinogen synthase differs from the described enzymes because of the lack of a metal requirement (155). The *hemB* gene encoding porphobilinogen synthase has been cloned and sequenced from *R. capsulatus* (96) as well as some other organisms such as rat (24), human (210), yeast (152) and other bacteria (61,81,113).

The next step in the tetrapyrrole pathway is the formation of urporphyrinogen III, the first cyclic tetrapyrrole and precursor to all hemes, chlorophylls and corrins (111). The conversion of porphobilinogen to uroporphyrinogen III is catalyzed by two enzymes: porphobilinogen deaminase encoded by the *hemC* gene and uroporphyrinogen III synthase encoded by the *hemD* gene (25,26). Porphobilinogen deaminase is responsible for the polymerization of four monopyrrole porphobilinogen molecules into the linear tetrapyrrole hydroxymethylbilane, with the help of a

covalently-bound dipyrromethane cofactor (109). Porphobilinogen deaminase is a monomer with molecular mass in the range of 33,000 to 44,000 Da (105) and has been purified from variety of sources including plants (87,105), mammalian sources (169), and bacteria (110,124). The porphobilinogen deaminase of *R. sphaeroides* is well characterized (106). It has been determined that the incorporation of the four substrate molecules into hydroxymethylbilane proceeds through covalently bound enzyme-intermediate complexes. Using the enzyme isolated from recombinant strains of *E. coli* it was shown that the deaminase contains a dipyrromethane cofactor linked to cysteine residue (86). The dipyrrole cofactor is synthesized from two porphobilinogen molecules (110). It remains covalently attached to the enzyme serving as a ligand for binding of four porphobilinogen substrate units to form the tetrapyrrole product (207). After cleavage of the bond between the cofactor and the product, the enzyme-bound cofactor is free to bind new substrate units and the tetrapyrrole product hydroxymethylbilane is released.

The *hemC* gene has been isolated and sequenced from a variety of prokaryotic and eukaryotic sources including bacteria (198,81), plant (211) and mammalian (14,165,189). The *hemC* gene from *R. capsulatus* has been cloned and sequenced recently (Canada and Biel, unpublished results). It has 49% identity to the *E. coli hemC* gene and 44% identity to the *Bacillus subtilis hemC* gene.

Hydroxymethylbilane has been shown to be the universal substrate for uroporphyrinogen III synthase. In the absence of uroporphyrinogen III synthase, hydroxymethylbilane spontaneously cyclizes forming uroporphyrinogen I in a nonenzymatic reaction. Uroporphyrinogen I is not a physiological metabolite. The biologically important metabolite uroporphyrinogen III is formed by cyclization of hydroxymethylbilane in an enzymatic reaction catalyzed by uroporphyrinogen III synthase.

Uroporphyrinogen III synthase is a monomeric enzyme with a molecular mass ranging from 28,000 Da to 38,500 Da (3,85). The enzyme has been purified from several sources (3,85,186,203). The *hemD* gene which codes for uroporphyrinogen III synthase has been cloned and sequenced from *E. coli* (1,109,174), *B. subtilis* (81) and other sources. The *hemD* gene has been found to be adjacent to the *hemC* gene (173) and they appear to form an operon where the *hemD* gene is under the control of the *hemC* promoter (108,148,174). The homology between the derived protein sequences of uroporphyrinogen III synthase from human and *E. coli* is very low, which is surprising considering the high degree of functional similarity between the deaminases.

The first branch point in the tetrapyrrole pathway is at uroporphyrinogen III. Methylation of that compound leads through several steps to the synthesis of siroheme and vitamin B-12. Siroheme functions as a prosthetic group of nitrite and sulfite reductases which generate the fully

reduced forms of nitrogen and sulfur used in biosynthesis (149-151). After the initial methylation of the tetrapyrrole ring of uroporphyrinogen III, siroheme is derived by a following oxidation reaction and chelation of Fe^{2+} into the ring. Vitamin B-12 functions as a cofactor of homocysteine methyltransferase (55). It is synthesized by a relatively small group of organisms including bacteria (121,144). Apparently vitamin B-12 is not synthesized by any eukaryotes and is neither required nor synthesized by higher plants (79). It has been shown that the photosynthetic bacteria *R. capsulatus* and *R. sphaeroides* do not have a vitamin B-12-independent route to synthesize methionine and, therefore, require the existence of the vitamin B-12 synthesizing pathway (35).

The major amount of uroporphyrinogen III is decarboxylated by the enzyme uroporphyrinogen III decarboxylase to form coproporphyrinogen III. The reaction proceeds with decarboxylation of all four acetate groups on the tetrapyrrole ring of uroporphyrinogen III to methyl groups (78). The enzyme can use both the biologically active isomer uroporphyrinogen III as well as uroporphyrinogen I as substrates. Uroporphyrinogen decarboxylase has been purified from *R. sphaeroides* (104) and *R. palustris* (125) as well as from yeast (64), avian (200) and mammalian sources (57,167,188). The enzyme exists as a monomer with a molecular mass of 42,000 Da (62) with the exception of the chicken enzyme which is a dimer with a native molecular mass of 79,000 Da (117). Unlike most decarboxylation reactions, uroporphyrinogen decarboxylase does not have a metal or coenzyme requirement. It is inhibited

by sulfhydryl group reagents and heavy metals such as Hg^{2+} and Cu^{2+} and most enzymes need a reducing agent for full *in vitro* activity (57,104,167,188). Based on the fact that point mutations which inactivate only part of the decarboxylation reaction cannot be found, it has been suggested that there is a single active site on the enzyme where all four decarboxylation reactions take place (36). Uroporphyrinogen decarboxylase is encoded by the *hemE* gene which has been cloned and sequenced from eukaryotic and prokaryotic sources such as human (168), yeast (71), *B. subtilis* (82), *E. coli* (158), and *R. capsulatus* (95).

In the next step of the tetrapyrrole pathway, the enzyme coproporphyrinogen III oxidase converts coproporphyrinogen III to protoporphyrinogen IX by oxidative decarboxylation of the propionic groups of rings A and B of coproporphyrinogen III to vinyl groups, thus producing protoporphyrinogen IX (160). Based on different genetic and biochemical studies, the presence of two distinct coproporphyrinogen III oxidase enzymes is suggested. One of them, designated HemF, requires molecular oxygen as an electron acceptor for the oxidative decarboxylation of coproporphyrinogen III and, therefore, functions under aerobic conditions (162,214). The other one, designated HemN, functions under anaerobic conditions and requires Mg^{2+}, methionine, ATP and either NAD^+ or $NADP^+$ for activity (178,192). Coproporphyrinogen III oxidase was purified to homogeneity from bovine liver (123) and the enzyme was shown to have molecular mass of 37,000 Da, as

determined by SDS-polyacrylamide gel electrophoresis. Since in earlier purification attempts by Yoshinaga and Sano (219) the molecular mass of the bovine enzyme was shown to be 72,000 Da by gel filtration, it was suggested that the mammalian enzyme is a homodimer. The yeast coproporphyrinogen oxidase was purified 150-fold by Poulson and Polglase (162) and the molecular mass was estimated by gel filtration to be 75,000 Da, which is similar to the bovine enzyme. Later, the enzyme was purified to homogeneity (31) and the gene coding for it (*HEM13*) was cloned and sequenced (222). It encodes a protein of 328 amino acids with a deduced molecular mass of 37,673 Da (31). Since the enzyme exists as a homodimer, its overall molecular mass corresponds to the one determined earlier by Poulson and Polglase (162). The expression of the yeast gene proved to be negatively regulated by changes in the levels of heme and oxygen (118,222,223). The *E. coli hemF* gene, encoding the oxygen-dependent coproporphyrinogen III oxidase, has been cloned using complementation of yeast *HEM13* mutant (202). It codes for a protein with a deduced molecular mass of 34,300 Da which shares a 43% identity to the *HEM13* gene product in yeast. One year later, the *E. coli hemN* gene coding for the oxygen-independent coproporphyrinogen III oxidase was cloned by complementation of a *hemF hemN* double mutant of *Salmonella typhimurium* (201). It encodes a 457 amino acid protein with an estimated molecular mass of 52,800 Da which has 92% identity to the *S. typhimurium hemN* gene and 35% identity to the *R.*

sphaeroides gene. Both the *hemF* (215) and the *hemN* (216) genes have been cloned from *S. typhimurium* and it was shown that either one can support heme synthesis under aerobic conditions (214). The *hemF* gene encodes a protein with deduced molecular mass of 34,000 Da. It possesses 44% identity to the yeast *HEM13* protein and 90% identity to the *E. coli hemF* protein (215). The *S. typhimurium hemN* gene codes for a protein with a molecular mass of 52,800 Da which has some identity (38%) to the *R. sphaeroides HemF* protein, but has no similarity to the *S. typhimurium hemF* gene product (216). Due to the accumulation of coproporphyrinogen III in *hemN* mutants and the fact that they are auxotrophic for hemin only when grown anaerobically, it was proposed that the HemN protein is an anaerobic coproporphyrinogen oxidase (214). A putative anaerobic coproporphyrinogen oxidase activity was detected originally by Tait in extracts of photosynthetically grown *R. sphaeroides* (192). Coomber et al. (41) recently reported the cloning and sequence analysis of a putative anaerobic coproporphyrinogen oxidase gene. However, the exact nature and mechanism of aerobic and anaerobic protoporphyrinogen IX synthesis remains to be elucidated.

The last biosynthetic step in the tetrapyrrole pathway which is common to both heme and bacteriochlorophyll is the formation of protoporphyrin IX by oxidative removal of six hydrogen atoms from protoporphyrinogen IX, a reaction catalyzed by the enzyme protoporphyrinogen IX oxidase. Initially, it was suspected that the reaction proceeds only as a simple non-enzymatic

chemical conversion, but Sano and Granick pointed out the involvement of an enzyme (172). Studying rat liver mitochondria, they showed that the rate of protoporphyrinogen IX oxidation was much higher, compared to the non-enzymatic reaction. Protoporphyrinogen IX oxidase has been purified to homogeneity from bovine and mouse liver (51,65,183), yeast (163) and the anaerobic bacterium *Desulfovibrio gigas* (120). The enzyme is a membrane-associated protein. The mammalian enzyme has been characterized as a monomer with a molecular mass of 65,000 Da, a little larger than the yeast enzyme (56,000 - 59,000 Da), which is bound to the inner mitochondrial membrane (47,51,183). The mammalian enzyme has a flavin adenine dinucleotide or a flavine mononucleotide associated with it (65,183). The bacterial enzyme purified from *D. gigas* is composed of three non-identical subunits with molecular masses of 12,000 Da, 57,000 Da, and 18,500 Da (120). The subunits are bound together via disulfide bonds (120). The purified enzyme has been shown to require 2,6-dichlorophenol-indophenol in vitro as an electron acceptor (120). In aerobic organisms, molecular oxygen is required for the reaction catalyzed by protoporphyrinogen oxidase because it serves as the electron acceptor (98-99). An oxygen-independent mechanism of protoporphyrinogen IX oxidation has been detected in anaerobically grown *E. coli* cells where nitrate and fumarate serve as electron acceptors (100-101). In addition, in photosynthetically grown *R. sphaeroides* cells protoporphyrinogen IX oxidation is closely linked to the respiratory electron

transport chain instead of using oxygen directly for oxidation (102). Direct indication of that was the decrease in enzyme activity by known respiratory inhibitors like hydroxylamine, cyanide and azide. When grown aerobically, protoporphyrinogen oxidase activity was detected, but at a rate which was half of that determined from photosynthetically grown cells. Protoporphyrinogen oxidase is encoded by the *hemG* gene which has been cloned, sequenced and characterized from two bacterial sources: *E. coli* (175) and *B. subtilis* (84). The cloned *hemG* gene from *E. coli* was able to complement an *E. coli hemG* mutant and the expressed product displayed strong protoporphyrinogen oxidase activity. A 546 nucleotides open reading frame has been identified and the predicted molecular mass for the HemG protein is 21,202 Da, much smaller than the plant, yeast and mammalian enzymes which display molecular masses in the range from 36,00 to 65,000 Da.

The second branch point in the tetrapyrrole pathway is at protoporphyrin IX, which is the last tetrapyrrole compound common both to heme and bacteriochlorophyll. If a ferrous iron is inserted into the ring of protoporphyrin IX, the pathway leads to synthesis of heme. However, if a magnesium ion is incorporated instead, it leads through several steps to the formation of bacteriochlorophyll. Ferrochelatase, also known as protoheme ferrolyase, is the terminal enzyme in the heme biosynthetic pathway which catalyzes Fe^{2+} insertion into protoporphyrin IX, yielding protoheme. Ferrochelatase activity was first described in 1956 by Goldberg et al. in

chicken erythrocytes extracts (74) and by Krueger et al. in duck erythrocytes extracts (127). The enzyme is ubiquitous and has been found in the whole range of organisms from bacteria to humans. In eukaryotes, the enzyme is located in the inner mitochondrial membrane, with the active site facing the matrix (103), whereas in prokaryotes it has a cytoplasmic membrane location except for the *B. subtilis* enzyme which has been reported to be a soluble enzyme (83). Interestingly, in higher plants ferrochelatase has two locations: one associated with mitochondria and the other one with chloroplasts (134). The insertion of Fe^{2+} into porphyrin can occur in the presence of excess ferrous iron as a spontaneous, non-enzymatic reaction which can also proceed under physiological conditions (116). However, the biological importance and requirement for ferrochelatase were clarified by Dailey and Lascelles (52) who showed the strict requirement for protoheme in a bacterial heme auxotroph which does not have ferrochelatase activity. Ferrochelatase was difficult to purify because of its instability, the requirement for solubilization with detergents and the formation of aggregates. Nevertheless, the homogeneous enzyme has been purified from diverse sources including bovine (196), mouse (49) and human livers (141), chicken erythrocytes (80), yeast (33), *R. sphaeroides* (44) and *B. subtilis* (84). All of the purified enzymes have comparable molecular masses in the range of 35,000 Da to 42,000 Da (33,49,196). The only exception is the *R. sphaeroides* enzyme which differs dramatically from the other ferrochelatases with a molecular

mass of 115,000 Da (44). It has been suggested that ferrochelatase is active as a monomer and that a high sodium cholate concentration can prevent its aggregation (49,83). However, Straka et al. (187) proposed that ferrochelatase could be biologically functional as a dimer. The actual ferrochelatase functional state remains to be unequivocally determined. Protoporphyrin IX is the physiological porphyrin substrate, but ferrochelatase exhibits a broad substrate specificity and can function with a variety of isomeric porphyrins such as deuteroporphyrin, mesoporphyrin and hematoporphyrin. It appears, however, that only the porphyrins which have uncharged substituents equal in size or smaller than hydroxyethyl at positions 2 and 4 on the A and B rings can be used as substrates (50). The natural metal substrate for ferrochelatase is ferrous iron (Fe^{2+}). Ferric iron (Fe^{3+}) is not a substrate (161). Some divalent metal ions such as cobalt (Co^{2+}) and zinc (Zn^{2+}) can be incorporated into the porphyrin ring as substrates (103), whereas other divalent metals such as manganese (Mn^{2+}), magnesium (Mg^{2+}), cadmium (Cd^{2+}), mercury (Hg^{2+}) and copper (Cu^{2+}) act as inhibitors (48). The most potent inhibitors of ferrochelatase are N-alkylporphyrins such as N-alkylprotoporphyrin, which has been shown to act by competitive inhibition (48,56). However, the precise model for the enzyme inhibition has not been conclusively elucidated. Sulfhydryl reagents such as iodoacetamide and N-ethylmaleimide have also been found to inhibit enzyme activity (45). *E. coli* ferrochelatase mutants were first recognized as being sensitive to light and

the locus responsible for that photosensitivity was identified and named *visA* (145). The cause for this photosensitivity was determined to be accumulation of protoporphyrin IX which cannot be converted to heme due to the lack of ferrochelatase activity (146,153). The ferrochelatase-encoding gene, named *hemH*, has been cloned and sequenced from variety of organisms such as mouse (194), human (154), cucumber and barley (147), *A. thaliana* (185), *S. cerevisiae* (73) as well as from bacterial sources: *E. coli* (145), *B. japonicum* (70), *B. subtilis* (82) and *R. capsulatus* (114). Amino acid sequence comparison between ferrochelatases from different sources revealed the carboxyl-terminal half of the protein to be highly conserved. Eukaryotic ferrochelatases, however, have an extra stretch of 30 to 50 amino acids at the carboxyl-terminus. The overall similarity between ferrochelatase sequences from mammalian sources such as mouse and human is 88%, whereas the sequence similarity between human and yeast ferrochelatases is 46% (129,154,194). All ferrochelatases cloned and sequenced so far are known to contain the following signature pattern: [LIVMF] (3)-x-S-x-H-[GS]-[LIVM]-P-x(4,5)-[DENK]-x-G-D-x-Y (9). This signature sequence contains a histidine residue which could be involved in the binding of iron. A [2Fe-2S] cluster identified recently appears to be a structural feature common to mammalian ferrochelatases (190). It is located in the C-terminus of mammalian ferrochelatases, but is absent in nonmammalian ferrochelatases. Dailey et al.

(47) proposed that the [2Fe-2S] cluster is essential for enzyme activity, but its exact biological role remains to be established.

The insertion of Mg^{2+} instead of Fe^{2+} into protoporphyrin IX is followed by methylation which produces magnesium protoporphyrin monomethyl ester. This compound is converted through several steps to bacteriochlorophyll.

Tetrapyrrole biosynthesis regulation

Resolving the complex regulatory mechanism directing tetrapyrrole synthesis has proven to be a challenging task. The end products of the tetrapyrrole pathway: heme, bacteriochlorophyll, siroheme and vitamin B-12, have very different functions in cell metabolism. Moreover, they are synthesized in dramatically different quantities depending on the growth mode of the organism. For example, during photosynthetic growth bacteriochlorophyll synthesis accounts for up to 98% of all tetrapyrrole synthesis. Most of the rest of the tetrapyrrole synthesized is heme and just trace amounts of vitamin B-12 and siroheme are made. If the *R. capsulatus* culture is switched to aerobic growth, bacteriochlorophyll synthesis immediately ceases, but the other three end products are synthesized at the same rate without any overproduction or accumulation of intermediates. This fact suggests a complex regulatory mechanism controlling tetrapyrrole biosynthesis which has been a focus of extensive research.

There are four environmental factors identified so far that have a role in regulation of tetrapyrrole synthesis in *R. capsulatus*: oxygen, heme, light and

c-type cytochromes . As demonstrated in the classic study by Cohen-Bazire et al.(40), synthesis of the photosynthetic apparatus is rapidly inhibited by molecular oxygen or increase in light intensity. It was proposed, that the regulated point is the oxidation of a component of the electron transport chain which could influence bacteriochlorophyll synthesis. Arnheim and Oelze (5) showed in a later study that oxygen and light independently influence bacteriochlorophyll levels in chemostat-grown cultures and cannot be considered as components of a single regulatory mechanism.

R. capsulatus with its variety of growth modes provides an attractive model for studying the regulation of tetrapyrrole synthesis. During aerobic growth, the cell membrane of *R. capsulatus* is morphologically and functionally similar to the cell membrane of most other Gram-negative bacteria. However, when the oxygen concentration is lowered below 2.5%, the cell develops an extensive intracytoplasmic membrane as a series of invaginations of the existing cytoplasmic membrane. The decrease in oxygen concentration induces the formation of the photosynthetic apparatus which is localized within the intracytoplasmic membrane, as well as induces the synthesis of bacteriochlorophyll and carotenoids. The photosynthetic apparatus is composed of three distinct pigment-protein complexes which carry out the conversion of light to chemical energy. The reaction center (RC) complex is the site of primary photochemical charge separation that ultimately leads to a cyclic flow of electrons through the electron transport chain, resulting in the

production of a transmembrane potential that drives the synthesis of ATP. There are two light-harvesting complexes: LHI (B870) and LHII (B800 - B850) which function to absorb visible and near-infrared radiation and to transfer this light energy with high efficiency to the reaction center complex. Each of these three complexes is composed of polypeptide subunits to which bacteriochlorophyll and carotenoids are noncovalently bound. In *R. capsulatus*, the bacteriochlorophyll genes (*bch*) encoding the enzymes that convert magnesium protoporphyrin to bacteriochlorophyll and the carotenoid genes (*crt*) encoding enzymes for the biosynthesis of carotenoids are clustered within a 46-kb region of the genome known as the photosynthetic gene cluster. That gene cluster includes also the *puh* and *puf* operons which contain genes encoding polypeptides of the light-harvesting complex I and the reaction center, respectively, but does not include the *puc* genes which encode the light-harvesting complex II polypeptides. The genes are arranged so that the *bch* and *crt* genes are tightly clustered in the central region which is flanked by the *puh* and *puf* operons (218). The entire 46-kb gene cluster of *R. capsulatus* has been recently sequenced by Hearst and Alberty (2). Identification of most of the genes required for bacteriochlorophyll biosynthesis in *R. capsulatus* was achieved by complementation of *bch* mutants with R'-plasmids carrying genes for various bacteriochlorophyll biosynthetic enzymes (140). A physical map showing the location of the bacteriochlorophyll and carotenoid genes was subsequently generated (197).

As mentioned earlier, a decrease in oxygen concentration induces the formation of the photosynthetic apparatus, whereas high levels of oxygen inhibit its formation. The exact mechanism of how oxygen controls bacteriochlorophyll synthesis has not yet been elucidated. There has been extensive research on that subject since oxygen was shown to repress bacteriochlorophyll synthesis in purple photosynthetic bacteria (40). Because of the lack of enzyme assay methods for most of the enzymes in the pathway from protoporphyrin IX to bacteriochlorophyll, an alternative method for measuring the transcription of the biosynthetic genes was used. Biel and Marrs (21) constructed fusions of the *lacZ* gene to various *bch* genes of *R. capsulatus*. In these fusion constructs, the *lacZ* gene was under the control of the *bch* gene promoters. The activity of these promoters was followed under different growth conditions by measuring the β-galactosidase activity. It was determined that transcription of the *bch* genes increased two- to three-fold with lowering of the oxygen tension from 23% to 2%. Other studies have confirmed these results by showing that the levels of mRNA for the *bch* genes of both *R. capsulatus* and *R. sphaeroides* are affected by oxygen (38,94). However, although transcription of the *bch* genes is regulated by oxygen two- to four-fold, this cannot account by itself for the dramatic down-regulation of bacteriochlorophyll synthesis in aerobically grown cultures of *R. capsulatus*.

In the search for factors that can influence bacteriochlorophyll biosynthesis, attention was focused on the *pufQ* gene located at the 5' end of

the *puf* operon in *R. capsulatus* and *R. sphaeroides*. The genes of the *puf* operon, including *pufQ*, are regulated by oxygen (179). More specifically, the transcription of *pufQ* is regulated 30-fold by oxygen (11). Genetic studies have indicated that the PufQ protein has a regulatory role in the bacteriochlorophyll biosynthetic pathway (10,122). However, the exact mechanism of that regulation is unclear. Deletion of the entire *puf* operon, while maintaining intact *bch* genes and *puc* operon, causes drastic reduction of the amount of bacteriochlorophyll-containing LHII complex (10). If a plasmid carrying the *pufQ* gene is introduced into such mutant strain, a normal level of LHII complex is observed. Bauer and Marrs used a fusion of the *pufQ* and *lacZ* genes to study the level of expression of *pufQ*. Based on the β-galactosidase activity, it was determined that the level of PufQ protein expression is directly proportional to the amount of bacteriochlorophyll synthesized when the gene fusion was introduced into *R. capsulatus* strain with a deletion of the *puf* operon. It is speculated that the PufQ protein functions as a carrier protein for intermediates of the entire magnesium branch of bacteriochlorophyll synthesis. On the other hand, recent results indicated that the PufQ protein may have a stimulatory effect early in the tetrapyrrole pathway (67).

In the effort to elucidate the mechanism of regulation of bacteriochlorophyll synthesis by oxygen, Rebeiz and Lascelles proposed a model which suggests that oxygen inhibits the magnesium chelatase and thus

causes increased heme production due to the conversion of a larger protoporphyrin IX pool by ferrochelatase (166). Heme would then feedback-inhibit aminolevulinate synthase which would reduce the carbon flow over the tetrapyrrole pathway to protoporphyrin IX and, therefore, inhibition of bacteriochlorophyll production would not cause overproduction of tetrapyrrole intermediates. According to this model, oxygen is involved in the regulation of two steps in the bacteriochlorophyll synthesis. Biel and Marrs demonstrated that oxygen regulates the synthesis of protoporphyrin IX (21). A *R. capsulatus bchH* mutant was grown under high and low oxygen tension and the accumulation of protoporphyrin was measured. The *bchH* mutant cannot synthesize bacteriochlorophyll due to the inability to chelate Mg^{2+} into protoporphyrin IX. Under low oxygen tension there was a drastic increase in the protoporphyrin levels, compared to high oxygen tension. This led to the suggestion that there is an oxygen-regulated step which directs the carbon flow over the common portion of the tetrapyrrole pathway.

The search for the step in the tetrapyrrole pathway that is regulated by oxygen started at the formation of aminolevulinate from succinyl CoA and glycine, catalyzed by aminolevulinate synthase. This was considered to be the first committed step for the tetrapyrrole pathway, but there is evidence that aminolevulinate is not committed solely to synthesis of tetrapyrroles and could be metabolized by another pathway. For example, Biel (unpublished results) showed that when *R. capsulatus* was grown in the presence of ^{14}C-

aminolevulinate, most of the radioactively labeled aminolevulinate (approximately 85%) was not incorporated into pigment, suggesting that aminolevulinate was converted to compounds other than tetrapyrroles. These results confirmed a previous study by Shigesada (182) showing that in *Rhodospirillum rubrum* ^{14}C-labeled aminolevulinate was incorporated into aminohydroxyvalerate, hydroxyglutarate and glutamate instead of tetrapyrroles. Moreover, an aminolevulinate dehydrogenase activity which converts aminolevulinate to aminohydroxyvalerate in the presence of NADPH was detected in *R. capsulatus* (Khanna and Biel, unpublished results).

Lascelles and Hatch (132) have reported that the formation of aminolevulinate is a rate-limiting step in *R. sphaeroides*. Moreover, *in vitro* inhibition of aminolevulinate synthase by heme was observed (29) which supported the model proposed by Rebeiz and Lascelles (166). However, in *R. capsulatus* there is no evidence that oxygen regulates the formation of aminolevulinate. There is no difference in the aminolevulinate synthase activity of *R. capsulatus* cultures grown under high and low oxygen tension (20). In addition, there is only two-fold change in the transcription of the *R. capsulatus hemA* gene in response to different oxygen tension, as determined in a transcriptional study using a *hemA-lacZ* fusion vector (212) which is not enough to explain the dramatic increase in tetrapyrrole synthesis during photosynthetic growth.

Addition of exogenous aminolevulinate to a *R. capsulatus bchH* mutant grown under high oxygen tension did not result in the accumulation of protoporphyrin, which suggested that the step regulated by oxygen is after the formation of aminolevulinate (20). Therefore, a *R. capsulatus bchH* mutant was grown under high and low oxygen tension in the presence of excess porphobilinogen. This resulted in accumulation of protoporphyrin not only under low oxygen tension, but also under high oxygen tension, suggesting that oxygen regulates the formation of porphobilinogen. However, neither the porphobilinogen synthase activity, nor the transcription of the *hemB* gene, as demonstrated by dot blot analysis and *hemB-cat* fusion experiments, is regulated by oxygen (96). Another possibility is that oxygen acts upon the degradation of porphobilinogen. Four molecules of porphobilinogen are required as a substrate in the next enzymatic step of the tetrapyrrole pathway catalyzed by porphobilinogen deaminase and uroporphyrinogen III synthase to form uroporphyrinogen III. Canada and Biel (unpublished results) have preliminary data that the ratio of porphobilinogen used to uroporphyrinogen formed varies two-fold when *R. capsulatus* cells are grown and assayed under strictly aerobic and anaerobic conditions. However, further investigation should determine if this is the true mechanism by which oxygen controls tetrapyrrole biosynthesis.

Regulation of bacteriochlorophyll synthesis by light was first demonstrated by Cohen-Bazire et al. (40) by showing lower levels of

bacteriochlorophyll in highly illuminated cultures. It was proposed that light is involved in the inhibition of bacteriochlorophyll synthesis. Biel and Marrs (21) demonstrated that light did not affect the transcription of the *bch* genes in *R. capsulatus* which, on the other hand are regulated by oxygen. Later, it was established that in *R. capsulatus* light intensity regulates not the bacteriochlorophyll synthesis, but the degradation of bacteriochlorophyll (17).

Another factor implicated in the regulation of tetrapyrrole synthesis is *c*-type cytochromes. Previous studies have suggested a link between *c*-type cytochromes and the carbon flow over the tetrapyrrole pathway, based on pigment accumulation in *R. capsulatus* strains with mutations in genes involved in *c*-type cytochromes biosynthesis (54,126). A *R. capsulatus* mutant, AJB530, which has a Tn5 insertion in the gene encoding cytochrome *c* synthetase and because of that is incapable of synthesizing *c*-type cytochromes, was isolated by Biel and Biel (18). Compared to the parental strain PAS100, AJB530 accumulates much lower amounts of bacteriochlorophyll, but excretes large amounts of coproporphyrin and protoporphyrin. It was proposed that *R. capsulatus* could increase the carbon flow over the tetrapyrrole pathway in an attempt to synthesize *c*-type cytochromes. However, the total porphyrin level, which includes the level of coproporphyrin, protoporphyrin and bacteriochlorophyll, was basically the same in PAS100 and AJB530 (93). This result indicated that the lack of *c*-

type cytochromes does not influence the carbon flow over the tetrapyrrole pathway.

One of the major factors influencing the regulation of tetrapyrrole synthesis is heme. *In vitro* inhibition of aminolevulinate synthase by heme was demonstrated by Burnham and Lascelles (29) in *R. sphaeroides*. When *R. capsulatus* or *R. sphaeroides* cultures were grown photosynthetically in iron-deficient media in order to limit heme production, large accumulation of coproporphyrin and Mg-protoporphyrin monomethyl ester was observed (Biel, unpublished results;42,130). Moreover, growth of *R. capsulatus* or *R. sphaeroides* cells in media supplemented with excess iron and in the presence of an inhibitor of ferrochelatase, N-methylprotoporphyrin, resulted in accumulation of Mg- protoporphyrin monomethyl ester, while the level of bacteriochlorophyll was not changed (Biel, unpublished results;92). These results suggest that heme-deficient cells have higher level of carbon flow over the tetrapyrrole pathway, which has been attributed to the lack of feedback inhibition of aminolevulinate synthase by heme (131).

The aim of this study was to further investigate the role of heme in the *R. capsulatus* regulatory scheme of tetrapyrrole biosynthesis. In order to achieve this goal, the *R. capsulatus hemH* gene was cloned using an *E. coli* Δ*hemH* mutant and sequenced. The gene was localized to the *R. capsulatus* chromosome. The *R. capsulatus hemH* gene was overexpressed by introducing it back into the parental *R. capsulatus* strain using a low-copy

number vector. Feedback inhibition of aminolevulinate synthase by heme was monitored both *in vivo* and *in vitro*. In order to distinguish whether heme and oxygen act by separate mechanisms to regulate tetrapyrrole synthesis, the effect of extra *hemH* on porphyrin accumulation was monitored under high and low oxygen tension.

MATERIALS AND METHODS

Strains and plasmids

The bacterial strains and plasmids used in this study are listed in Tables 1 and 2.

Media

E. coli was grown in L-broth (16) modified by omitting glucose and lowering the sodium chloride concentration to 0.5%. The *E. coli hemH* mutant ΔVisA was grown in L-broth supplemented with 15 μM hemin. *R. capsulatus* strains were grown in 0.3% yeast extract, 0.3% peptone (Difco Laboratories, Detroit, MI) (PYE) or in a minimal salts malate medium (RCV) (208). Solid media were supplemented with 1.5% agar (Difco). All bacterial strains were stored as one ml stocks in 10% glycerol at -80°C. Supplements, when needed, were added at the following final concentrations (μg/ml): ampicillin, 25; kanamycin, 10; tetracycline, 10 (*E.coli*) and 0.4 (*R. capsulatus*); streptomycin, 75; 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside, 40; isopropyl-β-D-thiogalactopyranoside, 5. When necessary, glucose and dimethyl sulfoxide were added to final concentrations of 0.25% and 20 mM, respectively.

Growth conditions

E. coli strains were grown at 37°C with shaking. *R. capsulatus* strains were routinely grown aerobically in the dark at 37°C with gentle shaking or

Table 1. Bacterial strains.

Strain Designation	Genotype	Reference
R. capsulatus		
PAS100	*hsd-1 str-2*	Taylor et al. (197)
SB1003	*rif-10*	Yen and Marrs (218)
AJB530	*hsd-1 str-2 ccl-1*	Biel and Biel (18)
E. coli		
MC1061	*Δ(ara-leu)7697 ΔlacX74 araD139 galU galK rpsL hsdR⁻ hsdM⁺*	Casadaban and Cohen (34)
NM522	*supE thi Δ(lac-proAB) hsd5/ F'[proAB⁺ lacI^q lacZΔM15]*	Gough and Murray (75)
HB101	*F⁻ara-14 proA2 lacY1 galK2 rpsL20 recA13 xyl-5 mtl-1 supE44 hsdS20(r⁻m⁻)*	Boyer and Roulland-Dissoix (27)
W3110	*F⁻*	Bachmann (8)
ΔVisA	*HfrC lac⁻$_{125\ amber}$ trp⁻$_{amber}$ su⁻ΔvisA*	Nakahigashi et al. (153)

Table 2. Plasmids

Plasmid	Characteristics	Short Description or Reference
pUC18	Ap^r	Vieria and Messing (204)
pRK404	mob^+, Tc^r	Ditta et al. (59)
pRK2013	mob^-, tra^+, Km^r	Ditta et al. (58)
pCAP122	Ap^r, *hemH*$^+$	3.1kb *R. capsulatus* PstI genomic fragment cloned into pUC18. (This study)
pCAP123	Ap^r, *hemH*$^+$	pCAP122 lacking the terminal HindIII fragment of the insert. (This study)
pCAP124	Ap^r, *hemH*$^-$	pCAP122 lacking the terminal EcoRI fragment of the insert. (This study)
pCAP125	Ap^r, *hemH*$^-$	pCAP123 lacking the terminal HindIII fragment of the insert. (This study)
pCAP126	Ap^r, *hemH*$^+$	1.7kb BamHI fragment from pCAP123 cloned into pUC18. (This study)
pCAP127	Ap^r, *hemH*$^+$	pCAP123 lacking the 0.3kb BamHI fragment next to the promoter. (This study)

(table con'd)

Plasmid	Characteristics	Short Description or Reference
pCAP134	Ap^r, *hemH*$^-$	0.6kb HindIII fragment of pCAP123 cloned into pUC18 in the opposite orientation. (This study)
pCAP135	Ap^r, *hemH*$^-$	0.6kb HindIII fragment of pCAP123 cloned into pUC18 in the same orientation. (This study)
pCAP140	Ap^r, *hemH*$^-$	1.0kb EcoRI fragment of pCAP123 cloned into pUC18 in the opposite orientation. (This study)
pCAP141	Ap^r, *hemH*$^-$	pCAP135 lacking the terminal BamHI fragment. (This study)
pCAP150	Tc^r, *hemH*$^+$	1.7kb BamHI fragment from pCAP123 cloned into pRK404. (This study)
pCAP151	Tc^r, *hemH*$^-$	Same as pCAP150 with insert in the opposite orientation. (This study)

without shaking, if used as the recipient for conjugation. When growth under specific oxygen tension was required, a ten ml overnight culture of *R. capsulatus* was subcultured into 100 ml of RCV with an initial Klett value of approximately 20 (red filter). The *R. capsulatus* culture was grown for two generations under high and low oxygen tension. In order to achieve high oxygen tension, the culture was subjected to sparging with compressed air supplemented with 3% oxygen. Low oxygen tension was achieved by sparging the culture with a mixture of 3% oxygen, 5% carbon dioxide, and 92% nitrogen. The cultures were incubated in a 37°C water bath. *R. capsulatus* was also grown photosynthetically in screw-capped tubes completely filled with RCV medium and incubated in front of 100 W light bulbs. *R. capsulatus* was grown anaerobically in the dark in RCV-filled screw-capped tubes, supplemented with 0.25% glucose and 20 mM dimethyl sulfoxide as a final electron acceptor. Growth was monitored by measuring the turbidity in a Spectronic 20 specrophotometer (Bausch and Lomb).

Chromosomal and plasmid DNA isolation

Chromosomal DNA from *R. capsulatus* was extracted from one ml overnight cultures grown in RCV medium using the Puregene DNA isolation kit (Gentra Systems, Inc., Minneapolis, MN). The cells were harvested by centrifugation at 16,000 x g for one minute and the pellet was suspended in 600 μl cell lysis solution. The samples were incubated at 80°C for five minutes to lyse the cells. RNase A solution (3 μl) was added to the cell lysate

followed by incubation at 37°C for 30 minutes. Tubes were cooled to room temperature and 200 μl protein precipitation solution was added followed by centrifugation at 16,000 x g for three minutes. The DNA-containing supernatant was transferred to a clean 1.5 ml tube containing 600 μl 100% isopropanol. After gentle mixing of the sample and centrifugation at 16,000 x g for one minute, the DNA was visible as a small, white pellet. The supernatant was poured off and the pellet was washed with 600 μl of 70% ethanol. After centrifugation at 16,000 x g for one minute, the ethanol was poured off and the DNA pellet was allowed to dry for 15 minutes. DNA hydration solution (100 μl) was added and the DNA was allowed to rehydrate overnight at room temperature.

Plasmid DNA minipreparations were obtained using the QIAprep Spin Plasmid Kit (QIAGEN Inc., Chatsworth, CA). The extraction protocol follows the basic steps of the alkaline lysis method of Birnboim and Doly (23). The plasmid DNA was recovered by adsorption to a silica-gel membrane in the presence of high salt which was subsequently washed away and the DNA was eluted with 50 μl to 100 μl of Tris-Cl buffer, pH 8.0.

Large-scale plasmid DNA preparations were obtained using the cesium chloride plasmid extraction method of Sidikaro and Nomura (184). One liter of culture was grown overnight in the presence of the appropriate antibiotic. The cells were harvested by centrifugation at 10,400 x g for five minutes in a Sorvall GSA rotor (Sorvall RC-5B Refrigerated Superspeed Centrifuge,

DuPont Instruments) and frozen at -80°C. Next, the cells were thawed on ice in 15 ml of 25% sucrose-50 mM Tris-Cl, pH 8.0. Lysozyme (30 mg) was added to the suspension followed by incubation for ten minutes on ice, addition of four ml of 0.3 M disodium ethylenediamine tetraacetic acid (EDTA), pH 8.0 and another ten minutes incubation on ice. Five milliliters of 5 M sodium chloride and 2.7 ml of 10% sodium dodecylsulfate (SDS) were slowly added while stirring the suspension followed by 20 minutes incubation on ice. After centrifugation at 27,000 x g for one hour in a Sorvall SS34 rotor, the supernatant was transferred to a teflon centrifuge tube and an equal volume of cold 20% polyethylene glycol (PEG) 8000-6% sodium chloride was added. Following incubation on ice for at least 20 minutes, the suspension was centrifuged at 3,020 x g for five minutes and the pellet was suspended in 3.5 ml of 10 mM Tris-Cl, pH 8.0-1 mM EDTA, pH 8.0 (TE). Next, an equal volume of chloroform was added and after mixing by inversion and centrifugation at 3,020 x g for five minutes, the aqueous layer was transferred to a 10 ml cylinder and the volume was adjusted to four ml with TE. Four grams of cesium chloride and 0.3 ml of ethidium bromide (10 mg/ml) were added. The suspension was centrifuged in a heat sealed ultracentrifuge tube overnight at 40,000 rpm in a Beckman VTi65 rotor (Beckman Instruments, Inc., San Ramon, CA) at 15°C. The plasmid-containing band was collected using a one ml syringe and a 20 gauge needle and transferred to a Corex tube. The ethidium bromide was removed by extracting the DNA two to three times with

an equal volume of isopropanol. The plasmid DNA was precipitated by addition of three volumes of cold ethanol, incubation at -80°C for 20 minutes and centrifugation at 9,750 x g for 15 minutes. The pellet was suspended in 0.3 ml deionized water and extracted with TE-saturated phenol followed by another ethanol precipitation in the presence of 0.3 M sodium acetate, pH 4.8. After centrifugation at 9,750 x g for 15 minutes, the pellet was dried under vacuum and suspended in 0.1 ml of sterile deionized water.

All chromosomal and plasmid DNA solutions were stored at -20°C. The concentration of DNA in each sample was determined by measuring the absorbance at 260 nm using a Lambda 3B spectrophotometer (Perkin-Elmer). At this wave length, one O.D. unit equals 50 μg DNA per ml.

Agarose gel electrophoresis

Agarose gel electrophoresis of chromosomal and plasmid DNA was performed using 0.7% agarose gels containing 0.5 μg/ml ethidium bromide. The electrophoresis was carried out in a Hoefer Gel Unit, Model HE-33 or a BRL Horizontal System, Model H5. A 50X TAE stock solution (1X TAE = 40 mM Tris-acetate, pH 8.0-2 mM EDTA, pH 8.0) was used as electrophoresis buffer and as a solvent for the gel preparation. The buffer was diluted to 4X for overnight gel runs at 25 volts or to 1X for short runs (1 to 2 hours at 90 volts). All DNA samples ware supplemented with one-tenth volume of loading buffer (40% sucrose-0.25% bromphenol blue). After the electrophoresis, the DNA bands were visualized with a Fotodyne short wave UV light box and

pictures were taken with a Polaroid camera, when needed. The size of the DNA fragments was estimated using molecular length markers of lambda DNA digested with HindIII.

Pulsed field gel electrophoresis

R. capsulatus SB1003 was grown overnight until $O.D._{660}$ of 0.8 was reached. Five milliliters of culture were harvested by centrifugation in a Sorvall SS34 rotor for six minutes at 3,020 x g and the pellet was suspended in five ml of 50 mM EDTA (pH 8.0). After another centrifugation for six minutes at 3,020 x g, the pelleted cells were resuspended in 0.5 ml of 50 mM EDTA (pH 8.0) and 0.5 ml of 1.6% low melting point agarose (Bethesda Research Laboratories, Inc., Gaithersburg, MD) cooled to 55°C. The suspension was transferred into a 50 cm length of sterile Nalgene tubing, 1/16 ID, 1/80D, 1/32 wall (Nalgene, Rochester, NY), the ends were clamped and it was allowed to solidify at room temperature. The cells were then extruded into five ml of 50 mM EDTA (pH 8.0) and incubated at room temperature for 30 minutes. The solution was replaced with five ml of 3% lauryl sarcosine, 0.2 M EDTA (pH 8.0), 5 mg proteinase K followed by overnight incubation at 55°C. The agarose plugs were solidified on ice for 30 minutes and rinsed twice with 50 mM EDTA (pH 8.0) for 30 minutes at room temperature. The solution was removed and 0.2 ml of 50 mM EDTA (pH 8.0)-1 mM phenylmethylsulfonylfluoride was added to inactivate the proteinase K. After incubation at room temperature for one hour, the plugs were rinsed twice with

50 mM EDTA (pH 8.0) for 30 minutes at room temperature. To prepare for restriction digestion, several one centimeter long plugs were placed in a microfuge tube with 0.1 ml of TE and incubated for 20 minutes on ice. The TE was removed and the plugs were incubated twice with 0.1 ml of the appropriate enzyme buffer solution for 30 minutes on ice. Next, the buffer solution was replaced with 0.1 ml of buffer solution containing the appropriate restriction enzyme and incubated for four hours at 37°C. After the enzyme digestion, the plugs were washed in 0.1 ml of 0.5 X TBE (45 mM Tris-45 mM boric acid-46 mM EDTA, pH 8.0) for 30 minutes on ice. The 1% agarose gel was loaded by placing the DNA-containing plug horizontally in the well and sealing it with 1% low melting point agarose. The electrophoresis was performed in a BioRad CHEF system (BioRad Laboratories, Hercules, CA) under the following conditions: voltage, 200V; temperature, 13°C; switching interval, five to thirty seconds; running time, 24 hours; buffer, 0.5 X TBE.

DNA recovery from agarose gels

DNA fragments were extracted from agarose gels following the method described by Chuang and Blattner (37). A 50 cm^2 piece of 3M paper (Whatman, Clifton, NJ) was cut into small pieces and placed in a tube filled with 40 ml of buffer (10 mM Tris-Cl, pH 7.4-1 mM EDTA, pH 7.4). The tube was shaken vigorously for five minutes and stored at 4°C. A hole was made with a 22-gauge needle in the bottom of a 0.5 ml centrifugation tube and enough paper slurry was added in order to completely cover the bottom of the

tube. Next, the tube was placed inside a 1.5 ml centrifugation tube and spun briefly to remove excess buffer. The tube was transferred to a new 1.5 ml centrifugation tube and DNA-containing agarose blocks were placed on top of the paper matrix. After centrifugation for ten minutes at full speed in an Eppendorf 5415 C microcentrifuge (Eppendorf, Madison, WI), the DNA sample in the 1.5 ml tube was dried down in a Speed Vac Concentrator (Savant Instruments, Inc., Farmingdale, NY), suspended in 10 μl of deionized water and ethanol precipitated. The DNA was stored suspended in sterile deionized water at -20°C.

Restriction enzyme digestion

DNA was routinely digested in a 10 μl volume using five to ten units of restriction enzyme per μg of DNA and 1 μl of the appropriate 10X reaction buffer. All restriction enzymes and buffers were supplied by New England Biolabs, Beverly, MA. The enzyme digestions were performed in a dri-bath incubator at the recommended incubation temperature for one to three hours.

Ligation

The DNA was prepared for ligation by precipitation with one-tenth volume of 3 M sodium acetate, pH 4.8 and three volumes of cold 95% ethanol. After incubation at -80°C for ten minutes, the sample was centrifuged for five minutes. The pellet was dried in a vacuum desiccator and suspended in sterile deionized water. The ligation reactions contained one to four μg of DNA, 2 μl of 5X T4 DNA ligase buffer, 1 μl (400 U) of T4 DNA ligase (New

England Biolabs, Beverly, MA), and water up to 10 μl final volume. The ligation reactions were incubated for three hours to overnight at 16°C for DNA fragments with cohesive ends or at 25°C overnight for blunt end DNA fragments.

Nick translation

Plasmid DNA was labeled by nick translation using a BRL nick translation kit (Life Technologies, Inc., Gaithersburg, MD). The reaction mixture contained 0.02 mM dGTP, dTTP and dCTP-0.05 M Tris-Cl (pH 7.8)-5 mM magnesium chloride-10 mM β-mercaptoethanol-3 μg nuclease-free bovine serum albumin-1 μg of DNA-20 μCi α^{32}P dATP (Amersham Life Science, Inc., Arlington Heights, IL). Next, 5 μl of DNA polymerase I / DNase I (20 units) were added and the reaction was incubated at 15°C for one hour. Following addition of five μl of stop buffer (0.3 M EDTA, pH 8.0), the reaction was passed through a Sephadex G-50 mini-spin column (Worthington Biochemical Corporation, Freehold, NJ) to remove the unincorporated nucleotides. Two μl of labeled plasmid DNA was spotted on a filter disc and five ml of CytoScint scintillation fluid (ICN Biomedicals, Costa Mesa, CA) was added. The counts per minute of the labeled DNA sample was quantified using a Liquid Scintillation Counter (Beckman model LS 6800).

Southern hybridization

After agarose gel electrophoresis, the gel was washed in 1.5 M sodium chloride-0.5 M sodium hydroxide for 20 minutes with slow shaking to denature

the DNA. For large DNA fragments (larger than ten kb), a prior wash in 0.25 N hydrochloric acid for ten minutes was required. Next, the gel was rinsed with distilled water and washed in 1.5 M sodium chloride-0.5 M Tris-Cl, pH 7.0 (neutralizing buffer) for 20 minutes with slow agitation. Following that, the gel was washed in 20X SSC (1X SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) for 20 minutes with slow shaking. The DNA fragments were transferred to a Nytran membrane prepared by brief rinse with distilled water and five minutes soaking in 20X SSC. The transfer was accomplished using the S&S Turboblotter and Blotting Stack Assembly (Schleicher & Schuell Inc., Keene, NH). The transfer time was three hours for small DNA fragments and overnight for large DNA fragments. After the transfer was complete, the blot was washed in 2X SSC for five minutes and baked at 80°C for one hour to immobilize the DNA. When necessary, the dried blots were stored in sealed plastic bags.

Blots were prehybridized at 65°C for one hour in 5 ml of the following prehybridization solution: 6X SSC-1% SDS-10X Denhardt's (one g Ficoll, one g polyvinylpyrrolidone, one g bovine serum albumin)-2.5 mg denatured salmon sperm DNA. The prehybridization was carried out in glass bottles rinsed with 2X SSC using the Hybaid Micro-4 Hybridization Oven (National Labnet Company, Woodbridge, NJ). Next, the prehybridization solution was replaced with 5 ml of 6X SSC-1% SDS-2X Denhardt's-0.5 mg denatured salmon sperm DNA-denatured probe (10^6 counts per minute of probe / ml).

The hybridization was carried out overnight at 65°C. The hybridization solution was then replaced with five ml of 2X SSC-1% SDS and the blots were washed for 30 minutes at room temperature. Next, they were washed in 0.5XSSC, 0.1% SDS for 30 minutes at room temperature and in the same solution for 30 minutes at 65°C. The blots were dried, taped to filter paper and used to expose Kodak XRP-5 X-ray film (Eastman Kodak Company, Rochester, NY) for one to two days. The film was developed using the standard manufacturer's procedure.

DNA sequencing

The sequencing templates were prepared using the Erase-a-Base System by Promega Corporation, Madison, WI. A series of nested deletions in the insert plasmid DNA were made with exonuclease III which specifically digests the DNA from a 5' overhang or blunt end while the 3' end is protected by generating a restriction fragment resistant to exonuclease III digestion. The steady rate of digestion by exonuclease III allowed obtaining of nested deletions by removing timed aliquots from the reaction sample. The DNA sequencing was performed with the CircumVent Thermal Cycle Dideoxy DNA Sequencing Kit (New England BioLabs, Beverly, MA) which follows the dideoxynucleotide chain termination method of Sanger et al. (171). Four reaction mixtures, each containing all four dNTPs and one of the four ddNTPs, were prepared. The DNA was labeled by incorporation of [α^{35}S] dATP. The samples were subjected to 25 cycles of denaturation at 95°C for one minute,

annealing at 55°C for one minute and chain extension at 72°C for one minute in the presence of the exonuclease-deficient Vent^R DNA polymerase from *Thermococcus litoralis*. The generated DNA fragments were denatured at 80°C for five minutes and separated by 6% polyarylamide urea gel electrophoresis. The electrophoresis was performed at 59°C in a Polar Bear Isothermal Electrophoresis System, Model S1SC (Owl Scientific, Woburn, NJ). Following the electrophoresis, the gel was placed on 3M Whatman paper of the same size and fixed in two liters of 20% ethanol-10% acetic acid for 30 minutes. The gel was dried in a BioRad Slab Dryer, model 483 (BioRad Laboratories, Hercules, CA) for two to four hours. The dried gel was placed in a film cassette with Kodak BioMax X-ray film (Eastman Kodak Company, Rochester, NY). The film was exposed for 48 to 60 hours.

Transformation

Chemical transformation

E. coli cells were grown with vigorous shaking to a cell density of 5 x 10^7 cells per ml ($O.D._{550}$=0.2) as monitored with a Spectronic 20 spectrophotometer (Bausch & Lomb). A sample of 1.5 ml of culture was harvested by centrifugation at 16,000 x g for one minute. The cell pellet was gently suspended in 1 ml of 100 mM morpholinopropane sulfonic acid, pH 6.5-50 mM calcium chloride-10 mM rubidium chloride and incubated on ice for 15 minutes. After centrifugation for one minute at 16,000 x g, the cell pellet was gently suspended in 0.2 ml of the same buffer and 3 μl of DMSO and up to 0.4

μg of DNA were added. The cell suspension was incubated on ice for 30 minutes followed by a heat shock in a 44° C water bath for 90 seconds. The suspension was diluted with 1 ml of L-broth and incubated without shaking at 37°C for 60 minutes. One-tenth ml of cells was plated on a selective medium. The described chemical transformation procedure is based on the procedure developed by Kushner (128).

Electroporation

E. coli cells were grown in L-broth until $O.D._{660}$ in the range of 0.5 to 1.0 was reached. Ten to twenty ml of culture was transferred to a sterile centrifuge tube and chilled on ice for ten minutes. After centrifugation at 5,900 x g in a Sorvall SS34 rotor for eight minutes, the pellet was suspended in 10 ml of sterile ice cold water, chilled on ice for five minutes and centrifuged at 5,900 x g for eight minutes. The pellet was washed with another 10 ml of sterile ice cold water followed by two consecutive washes in 10 ml sterile ice cold 10% glycerol. The cells were suspended in 0.1 ml to 0.2 ml of sterile ice cold 10% glycerol. Forty microliters of the freshly prepared competent cells and up to 3 μg of DNA were transferred to a sterile 0.2 cm cuvette and mixed. The cells were subjected to electroporation in a BioRad Gel Pulser (BioRad Laboratories, Hercules, CA) using the following settings: 2.5 kV, 25 μF, and 200 ohms. The cell suspension was transferred immediately to a tube with 2 ml of L-broth. The L-broth was supplemented with 15 μM hemin when the *hemH* deletion mutant ΔVisA was used. After incubation at 37°C for 60

minutes in a roller drum, an aliquot of cells was plated out on a selective medium.

Conjugation

The *R. capsulatus* recipient strains were grown in 10 ml of PYE medium at 37°C overnight without shaking. *E. coli* donor strains as well as the *E. coli* strain HB101 carrying the helper plasmid pRK2013 were grown in 10 ml of L-broth supplemented with appropriate antibiotics overnight at 37°C with shaking. One milliliter of the *R. capsulatus* recipient culture was mixed with 0.2 ml of each of the *E. coli* cultures in a sterile tube. After centrifugation, the pellet was washed with 1 ml of RCV broth to eliminate any traces of salt from the L-broth which would inhibit the growth of *R. capsulatus* strains. The cells were harvested, suspended in 0.1 ml RCV broth and transferred to a nitrocellulose disc placed on a PYE plate. After incubation at room temperature for four hours to overnight, the nitrocellulose disc was transferred to a RCV plate supplemented with appropriate antibiotics. The cells were spread with 0.1 ml of RCV broth and the plate was incubated at 37°C for two to three days to select for exconjugants.

Protein determination

Protein determination by dye binding

Protein concentration in cell extracts was determined using the BioRad Protein Assay (BioRad Laboratories, Hercules, CA) based on the method of Bradford (Bradford, 1976). Bovine serum albumin (BSA) was used to create a

standard curve. Four dilutions of between 10 μg and 70 μg BSA were placed in a test tube. The cell extract to be tested for protein concentration was placed in a separate tube. Deionized water was added to all tubes to bring the total volume to 4 ml. Next, 1 ml of BioRad Dye Reagent Concentrate was added to each tube and the mixture was vortexed. The samples were incubated at room temperature for five minutes and the absorbance at 595 nm was measured. The protein concentration in the tested cell extract was determined by comparing its absorbance to the BSA standard curve.

Lowry protein determination

The Lowry protein determination method (135) was used in a modified version to estimate the protein concentration in cell samples used for bacteriochlorophyll measurements. The cell pellet was dissolved in one ml of 0.2 N sodium hydroxide and boiled for two minutes. One tenth ml of that sample was transferred to another tube and the volume was adjusted to 0.5 ml with deionized water. Bovine serum albumin (BSA) was used as a standard. Five tubes containing from 0 mg to 0.4 mg BSA per ml were supplemented with 0.1 ml 0.2 N sodium hydroxide and the final volume was adjusted to 0.5 ml with deionizied water. Five milliliters of reagent A (1 ml 1% copper sulfate-1 ml 1% sodium potassium tartarate-98 ml 2% sodium carbonate in 0.1 N sodium hydroxide) was added to each 0.5 ml sample tube. After incubation at room temperature for ten minutes, 0.5 ml of reagent B (1 N Folin's reagent) was added to each tube. Following a 30 minutes incubation

at room temperature, the absorbance at 660 nm was measured and the protein concentration of the tested samples was determined by comparison of their absorbance to the BSA standard curve.

Sonication

R. capsulatus was grown in 200 ml of either RCV or PYE medium until late log phase was achieved. The cells were harvested by centrifugation at 10,400 x g for ten minutes. Cell pellets were suspended in 2 ml of 0.1 M Tris-Cl, pH 7.5 and sonicated three to four times for 20 seconds each with one minute cooling periods using a Heat Systems Ultrasonic Sonicator, model W-220 (Ultrasonics, Inc.). The cell suspension was centrifuged in a Sorvall SS34 rotor at 27,000 x g for 20 minutes and the cell extract was transferred to another tube and stored at 4°C.

Quantitation of coproporphyrin and protoporphyrin

The concentration of coproporphyrin and protoporphyrin in *R. capsulatus* cultures was estimated as described (113). Coproporphyrin and protoporphyrin were extracted from up to 1 ml of cell culture with 3 ml of ethyl acetate-acetic acid (3:1). The top layer was transferred to another tube and extracted with 2 ml of 3 N hydrochloric acid. The bottom hydrochloric acid layer was removed to another tube and saved. The top layer was extracted again with 2 ml of 3 N hydrochloric acid and both hydrochloric acid layers were combined. The fluorescence of the samples was measured using excitation wavelength of 407 nm and emission wavelength of 605 nm in a

Perkin-Elmer LS-3 Fluorescence Spectrophotometer (Perkin-Elmer Corporation, Norwalk, CT). The fluorescence value was used to determine the porphyrin concentration (coproporphyrin and protoporphyrin) by comparison to a standard curve prepared using coproporphyrin and protoporphyrin standards (Porphyrin Products, Logan, UT).

Quantitation of bacteriochlorophyll

The bacteriochlorophyll concentration in *R. capsulatus* cultures was determined by harvesting 1 ml of cell culture, suspending the cell pellet in 1 ml of deionized water and extracting the sample with an equal volume of acetone-methanol (7:2). After incubation in the dark for 15 minutes, the suspension was centrifuged and the absorbance of the supernatant was measured at $O.D._{770}$ in a Perkin-Elmer Lambda 3B UV/VIS Spectrophotometer (Perkin-Elmer Corporation, Norwalk, CT). The pellet was saved for protein concentration estimation by the modified Lowry procedure. The bacteriochlorophyll concentration of the sample was determined in nanomoles based on the method of Clayton (39).

Ferrochelatase assay

R. capsulatus cell extracts were prepared by sonication, as described previously. The assay mixture contained 250 μl to 750 μl of cell extract, 50 μl of 1 M Tris-Acetate, pH 8.2, 50 μl of 0.1 M dithiothreitol, 50 μl of 4 mM ferrous ammonium sulfate, and 100 μl of freshly prepared deuteroporphyrin. The tube was kept wrapped in foil to protect the light sensitive deuteroporphyrin. After

incubation at 37°C for 30 minutes, the enzyme reaction was stopped by addition of 500 μl of 50 mM iodoacetamide. Following addition of 250 μl pyridine and 250 μl 1 N sodium hydroxide to obtain a pyridine hemochrome, the mixture was split into two cuvettes. A few grains of sodium dithionite were added to the sample cuvette to reduce the sample and the difference spectrum was recorded from 575 nm to 500 nm using a Kipp & Zonen BD40 chart recorder. Based on the difference between the oxidized and reduced spectra, the ferrochelatase activity was calculated as nanomoles deuteroheme formed per minute per mg of protein. The ferrochelatase assay is based on the method developed by Dailey (44).

Aminolevulinate synthase assay

The aminolevulinate synthase assay is based on the procedure described by Burnham (30). *R. capsulatus* cell extracts were prepared as described in the sonication section. The assay mixture contained 75 μl of cofactor mixture (2 ml 0.2 M ATP, pH 7.0-1.75 ml 0.01 M CoA-1.35 ml 0.01 M pyridoxal phosphate-2 ml deionized water), 175 μl substrate mixture (5 ml 1 M glycine-5 ml 1 M succinate, pH 7.0-5 ml 0.1 M $MgCl_2$-2.5 ml 1 M Tris-Cl, pH 7.5), and 250 μl of crude cell extract and deionized water. The blank contained the same ingredients except that the substrate mixture was substituted with deionized water. After incubation at 37°C for 30 minutes, the enzyme reaction was terminated by addition of 250 μl of 10% trichloracetic acid. The precipitated proteins were removed by centrifugation for two

minutes. The supernatant was transferred to another tube to which 1 ml of sodium acetate, pH 4.7 and 25 μl of 2,4-pentanedione were added. The tube was heated at 100°C for 15 minutes. Next, it was cooled down to room temperature and 1.75 ml of freshly prepared Erhlich's reagent (0.5 g p-dimethylaminobenzaldehyde-21 ml glacial acetic acid-4 ml 70% perchloric acid) was added. Following incubation at room temperature for 20 minutes, the optical density at 556 nm was determined. The aminolevulinate synthase specific activity was determined as nanomoles aminolevulinate formed per hour per mg protein.

In vitro inhibition of aminolevulinate synthase by heme and protoporphyrin IX

Aminolevulinate synthase assays were performed as described above. Increasing concentrations of heme or protoporphyrin IX (1 to 125 μM) were added to separate reaction mixtures before incubation. In addition, separate blanks were prepared for each reaction mixture. Each blank contained the same concentration of heme or protoporphyrin IX as the corresponding reaction mixture as well as the same ingredients for the aminolevulinate synthase assay, except the substrate mixture which was substituted for deionized water. Aminolevulinate synthase specific activity for each heme-containing or protoporphyrin IX-containing reaction was determined based on the corresponding blank. The percent inhibition was determined based on the

aminolevulinate synthase activity in a control reaction containing no heme or protoporphyrin IX. The standard error for each point was also calculated.

RESULTS

Cloning of the *R. capsulatus hemH* gene

Chromosomal DNA from *R. capsulatus* strain PAS100 was extracted. Restriction digestion of the DNA was performed using three different restriction enzymes: EcoRI, HindIII, and PstI, in separate reactions. *R. capsulatus* PAS100 libraries were prepared by ligating chromosomal DNA from each reaction into pUC18. The expression vector pUC18 is a 2,686 base pair long high copy number plasmid carrying the *lac* promoter and the β-lactamase gene which confers ampicillin resistance (204). Each *R. capsulatus* library was transferred by electroporation into ΔVisA, an *E. coli* Hem^- mutant. ΔVisA has a deletion of the *hemH* gene and because of that requires exogenous hemin for growth in the absence of a fermentable carbon source. Complemented ΔVisA colonies were selected for by plating each transformation mixture on L-agar supplemented with ampicillin and without hemin. An ampicillin-resistant colony that no longer required hemin for growth was isolated when using the PstI library. No colonies were recovered from the EcoRI and the HindIII libraries. The Hem^+, ampicillin-resistant cells were grown in L-broth containing ampicillin for plasmid isolation. The extracted plasmid was designated pCAP122 (Fig. 2). The plasmid has a 3.1 kb PstI fragment containing the *R. capsulatus hemH* gene. Plasmid DNA from pCAP122 was used to transform ΔVisA. All transformants were

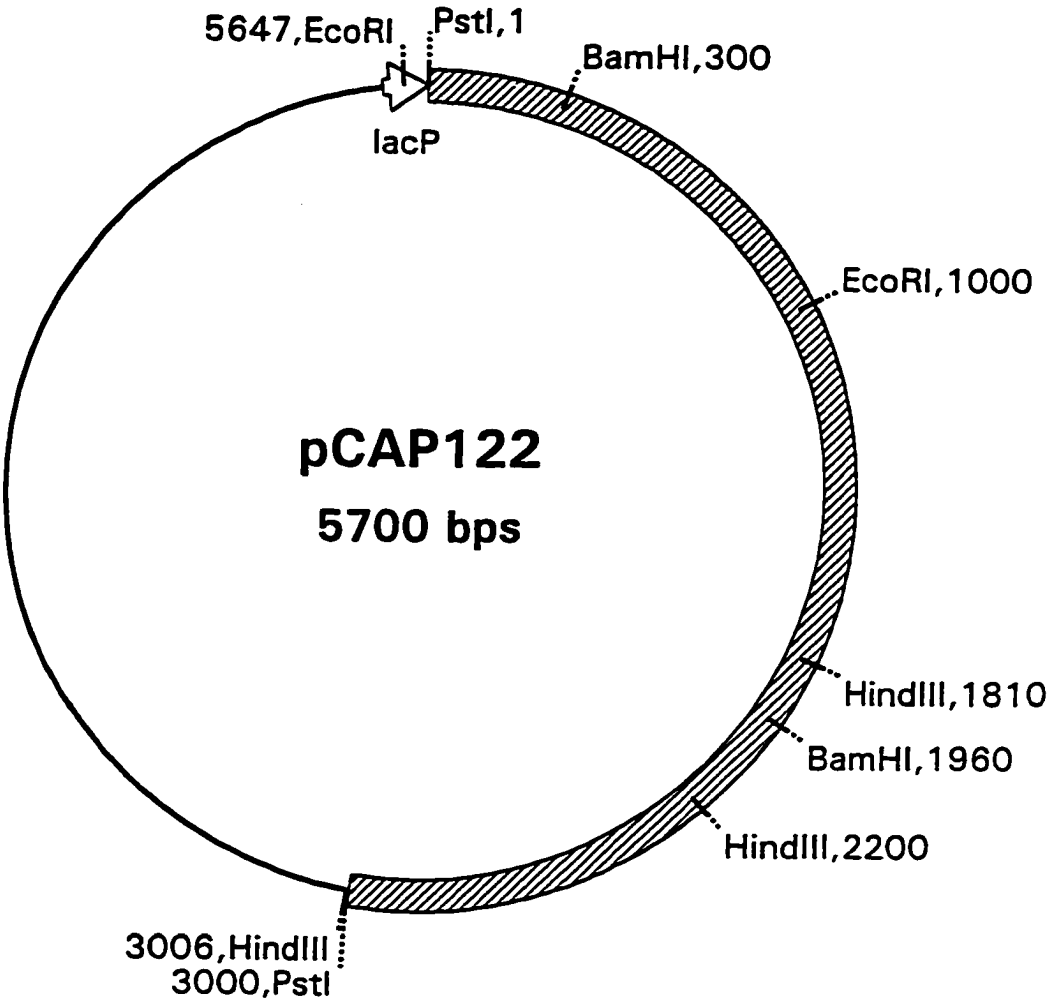


Figure 2. The ΔVisA complementing plasmid pCAP122.

ampicillin-resistant and Hem$^+$, which demonstrated that pCAP122 carries the *hemH* gene.

Restriction mapping of the *hemH* region

In order to localize the *R. capsulatus hemH* gene, a series of deletions in the PstI insert of pCAP122 were made (Fig. 3). The first derivative, designated pCAP123, lacks the terminal HindIII fragment, which extends from basepair 2200 to basepair 3006. Transformation of ΔVisA with pCAP123 resulted in complementation of the heme deletion mutant suggesting that the *hemH* gene was intact in the insert of pCAP123 and was not disrupted by the HindIII fragment deletion.

In another derivative, designated pCAP124, the terminal EcoRI fragment, which extends from basepair 1 to basepair 1000, was deleted. This clone did not complement ΔVisA, suggesting that the *hemH* gene or part of it was located in the deleted EcoRI fragment.

The plasmid pCAP125 was formed by deleting the terminal HindIII fragment of pCAP123 (basepair 1810 to basepair 2200). The plasmid was transferred by electroporation into ΔVisA. This clone did not complement ΔVisA, suggesting that a part of the *hemH* gene was in that HindIII fragment.

Finally, pCAP126 was created by subcloning the BamHI fragment from the pCAP122 insert into pUC18. This clone complemented ΔVisA. This located the *hemH* gene of *R. capsulatus* to a 1.7 kb BamHI fragment.

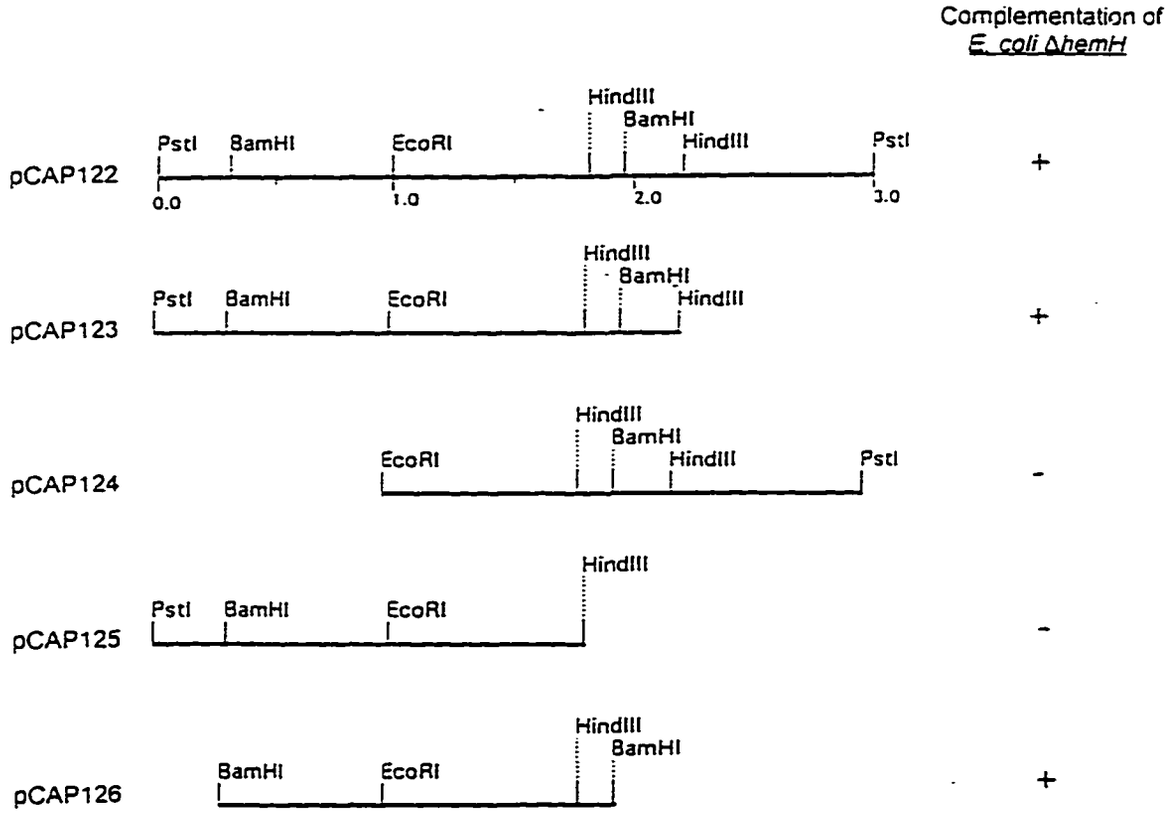


Figure 3. Restriction map of *R. capsulatus hemH* derivatives. Distances are expressed in kilobases.

Ferrochelatase enzyme assay

In order to confirm the cloning of the *R. capsulatus hemH* gene, a ferrochelatase assay was performed. Ferrochelatase is the enzyme coded for by the *hemH* gene (146). Ferrochelatase activities were determined in a Hem^+ *E. coli* strain (W3110), ΔVisA, and ΔVisA carrying pCAP123. As expected, no ferrochelatase activity was detected in the *hemH* deletion mutant ΔVisA. The presence of pCAP123 in ΔVisA increased ferrochelatase activity to a level almost three times higher than that in the Hem^+ strain W3110. The results are summarized in Table 3.

Chromosomal localization of the *R. capsulatus hemH* gene

The location of the *hemH* gene on the chromosome of *R. capsulatus* was determined using the *R. capsulatus* strain SB1003 and the physical map of its genome. The map was constructed by digesting the chromosome of SB1003 with the restriction enzymes AseI and XbaI in separate reactions and overlapping the resulting fragments (68). SB1003 chromosomal DNA was digested with the restriction enzymes AseI and XbaI, as described in Materials and Methods. Following separation of the fragments by pulsed field gel electrophoresis, they were transferred to a Nytran membrane. The *hemH* clone pCAP123 was ^{32}P-labeled via nick translation and used as a probe in a Southern hybridization. The *hemH* gene was localized to AseI fragment 1 (650 kb) and XbaI fragment 2 (490 kb). The results from the Southern hybridization

Table 3. Ferrochelatase enzyme assay.

Strains	Specific activity[a]
W3110	30
ΔVisA	0.17
ΔVisA + pCAP123	86

[a] Specific activity is expressed as pmoles of deuteroheme formed per minute per mg of protein.

are represented in Fig. 4. The location of the *hemH* gene as well as the location of the other *hem* genes of *R. capsulatus* mapped to the SB1003 physical chromosomal map is shown in Fig. 5.

Sequencing of the *R. capsulatus hemH* gene

A series of nested deletions in the insert of pCAP123 (*hemH*) were created. These clones were used for sequencing of *hemH* in both directions. The *R. capsulatus hemH* gene is contained within an open reading frame of 1056 nucleotides (Fig. 6). The *R. capsulatus* consensus codon preference table (220) was used to confirm the correct *hemH* open reading frame. According to the *R. capsulatus hemH* codon preference plot (Fig. 7), the longest open reading frame in the third panel corresponds to *hemH*. There are eight rare codons present. There are three potential start codons beginning at positions 14, 176 and 222 in the correct reading frame in this region of the sequence. Based on the codon preference plot, the presence of a putative Shine-Delgarno sequence and homology to the *E. coli* and *B. subtilis* amino acid sequences, the ATG codon beginning at position 222 is the most likely start codon for the *hemH* gene. The open reading frame encodes a putative protein of 351 amino acids with a predicted molecular mass of 46,210 Da which is similar to the molecular mass of other ferrochelatases (43,129,147,154,194). It differs from the value of 115,000 Da reported for the ferrochelatase of another *Rhodobacter* species, *R. sphaerodes* (44), which does not match the molecular mass of any eukaryotic or prokaryotic

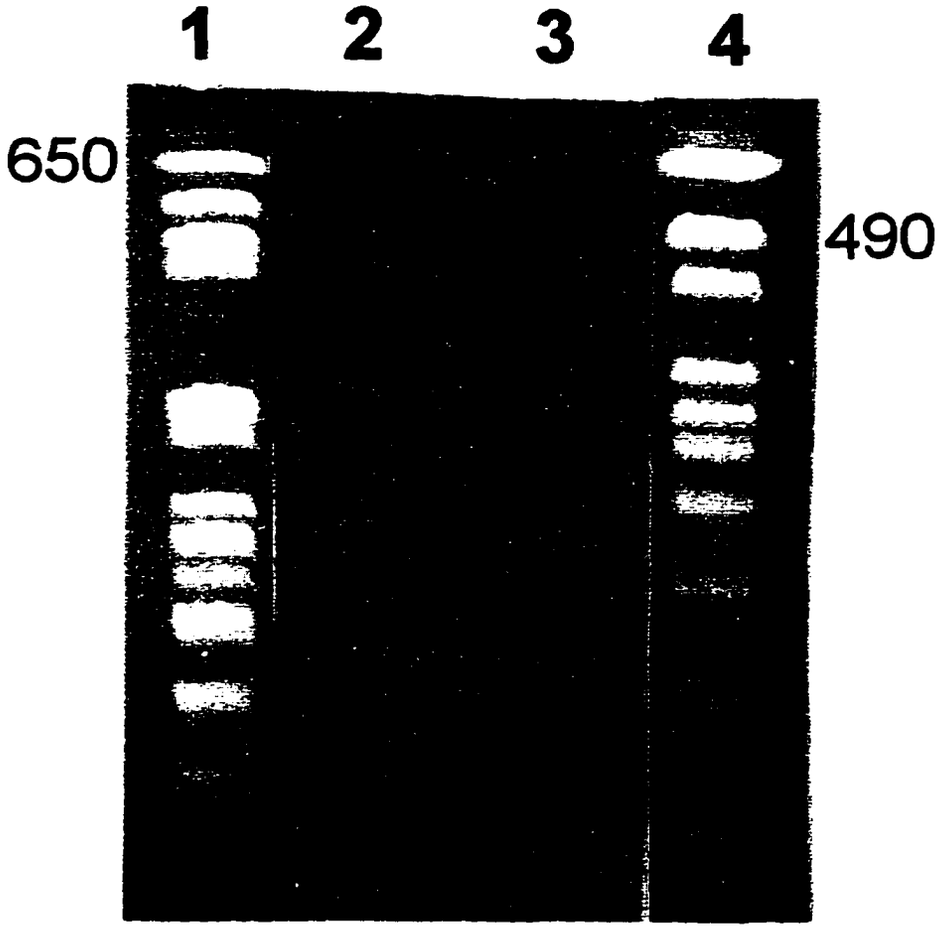


Figure 4. Southern hybridization of *R. capsulatus* SB1003 chromosomal DNA with pCAP123 (*hemH*)

1 - SB1003 chromosomal DNA digested with AseI
2 - pCAP123 hybridized to AseI fragment 1 (650 kb)
3 - pCAP123 hybridized to XbaI fragment 2 (490 kb)
4 - SB1003 chromosomal DNA digested with XbaI

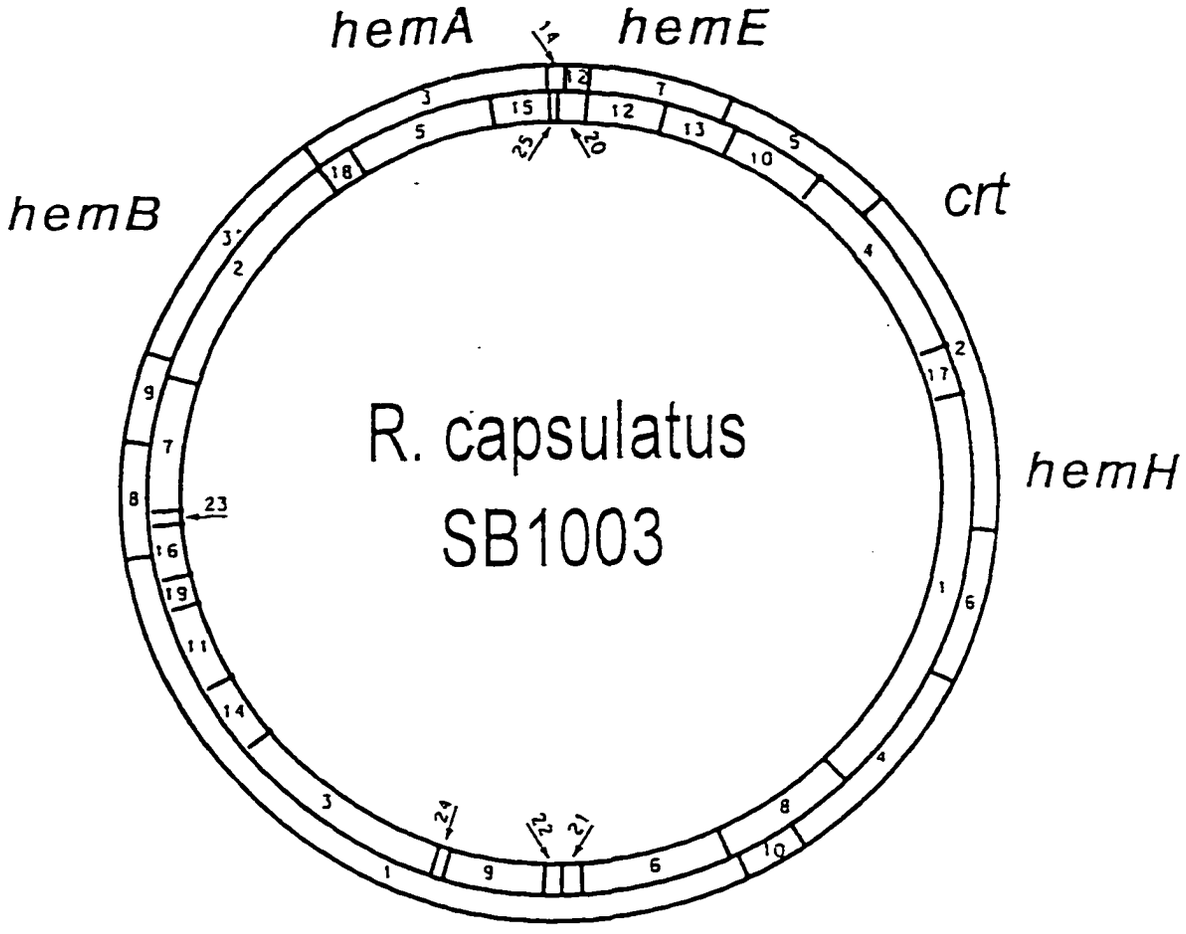


Figure 5. Location of *hemH*, *hemA* (96), *hemB* (97), *hemE* (95), and *crt* genes on the physical map of *R. capsulatus* SB1003. The map was constructed by Fonstein et al (68).

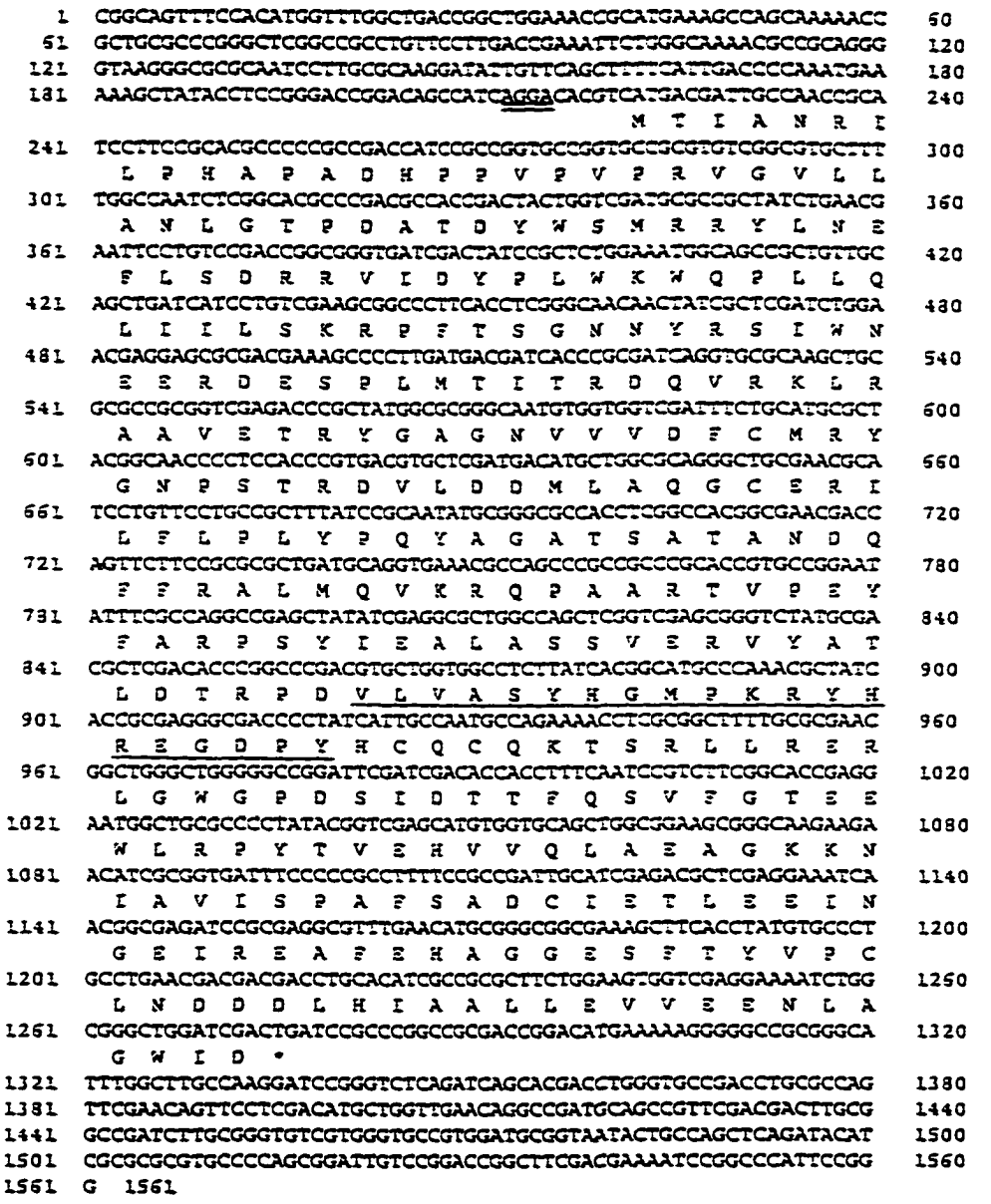
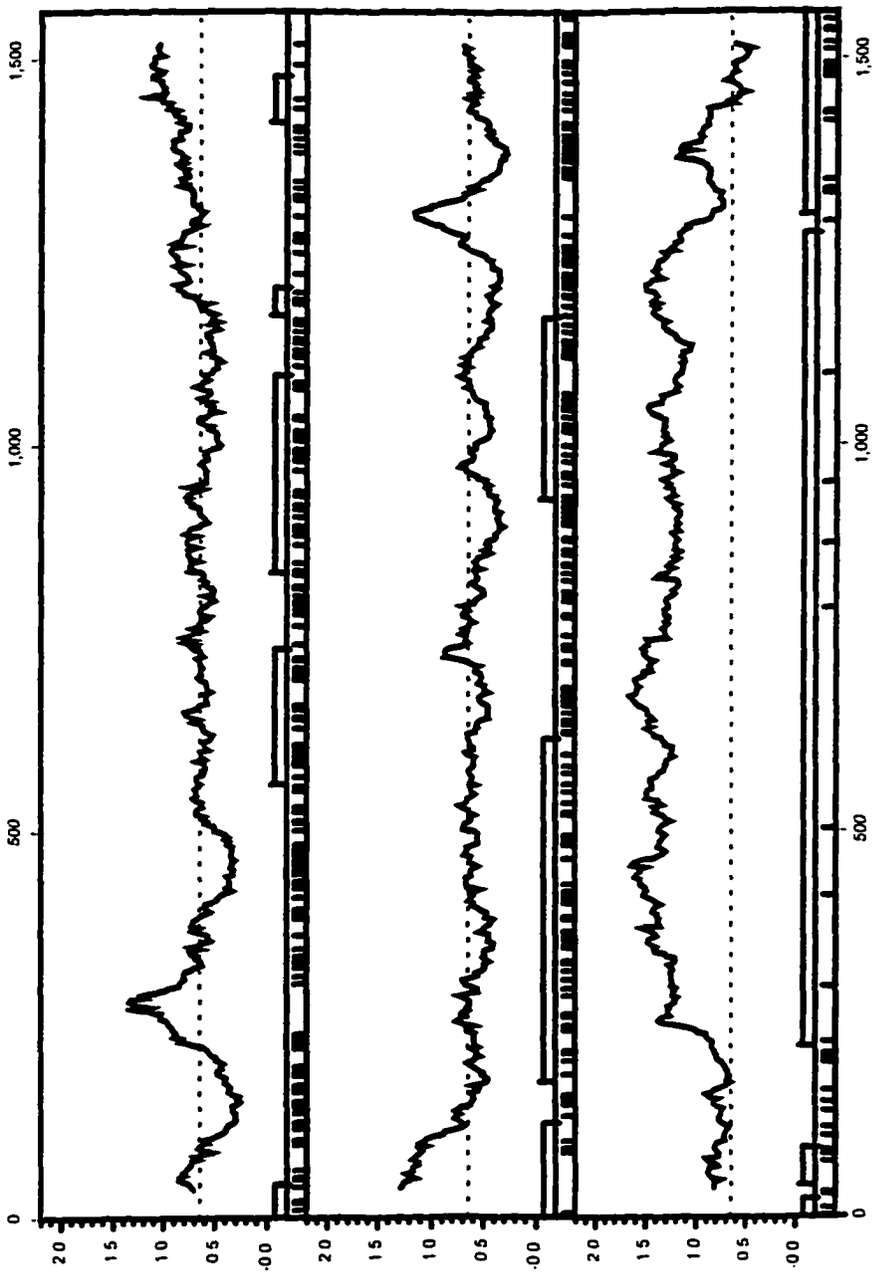


Figure 6. Nucleotide sequence of the *R. capsulatus hemH* gene. The putative Shine-Delgarno sequence is underlined with a double line and the consensus ferrochelatase signature sequence is underlined with a single line.

Figure 7. Codon Preference Plot of the nucleotide sequence containing the *R. capsulatus hemH* gene.

The open reading frame corresponding to *hemH* is shown in the bottom panel. The dashed line in the middle of all three panels represents the codon threshold. Open reading frames are indicated as uninterrupted boxes at the bottom of each panel. Rare codons are represented by vertical lines at the bottom of each panel.



ferrochelatase purified so far. The amino acid sequence has 41% identity and 59% similarity to the *E. coli* ferrochelatase and 21% identity and 44% similarity to the *Bacillus subtilis* ferrochelatase (82). The consensus ferrochelatase signature sequence [LIVMF](3)-x-S-x-H-[GS]-[LIVM]-P-x(4,5)-[DENK]-x-G-D-x-Y (9) was confirmed in the *R. capsulatus hemH* sequence, beginning at Val^{214}, with the exception of Lys^{228} which is substituted by an arginine.

Overexpression of the *hemH* gene in *R. capsulatus*

In order to study the effect that heme might have on regulating tetrapyrrole biosynthesis in *R. capsulatus*, the levels of heme were increased *in vivo* by providing additional copies of the *hemH* gene. The 1.7 kb BamHI fragment from pCAP126 carrying the *hemH* gene was subcloned into the broad host-range vector pRK404 (59) which carries the tetracycline resistance marker and can replicate in *R. capsulatus*. This construct, pCAP150, was moved into the *R. capsulatus* strain PAS100 (195) by conjugation with selection for tetracycline resistance. Both PAS100 and PAS100(pCAP150) were grown in 200 ml of RCV medium in 500 ml flasks with slow shaking until late exponential growth phase was reached. Interestingly, the cultures differed markedly in color. The culture of PAS100(pCAP150) had a light orange color, whereas the culture of the parental strain PAS100 was dark red. This suggested that excess ferrochelatase influences the level of pigments in PAS100. Monitoring the absorbance spectra in extracts of the cultures revealed that the level of both light-harvesting complexes was much lower in

PAS100(pCAP150), compared to the parental strain PAS100 alone (Fig. 8). This suggested that bacteriochlorophyll production decreases in the presence of excess ferrochelatase.

In order to understand better the role of excess ferrochelatase in *R. capsulatus*, the levels of bacteriochlorophyll and porphyrin (coproporphyrin and protoporphyrin) as well as the specific activity of ferrochelatase and aminolevulinate synthase were measured in PAS100 and PAS100(pCAP150), as described in Materials and Methods. The results are summarized in Table 4. As expected, ferrochelatase activity was three fold higher in PAS100(pCAP150), compared to PAS100 alone, due to the presence of additional copies of the *hemH* gene. Interestingly, the presence of pCAP150 decreased the aminolevulinate synthase activity, indicating that heme probably feedback inhibits aminolevulinate synthase in *R. capsulatus*. In addition, PAS100(pCAP150) had lower bacteriochlorophyll levels than PAS100, which confirmed the results from the absorbance spectra (Fig. 8).

The combined level of coproporphyrin and protoporphyrin was much lower in PAS100(pCAP150) than in the parental strain, as shown in Table 4. As a control, the vector pRK404 was transferred into PAS100 by conjugation. The plasmid had no effect on the ferrochelatase and aminolevulinate synthase enzyme activity, porphyrin accumulation, and bacteriochlorophyll levels (data not shown).

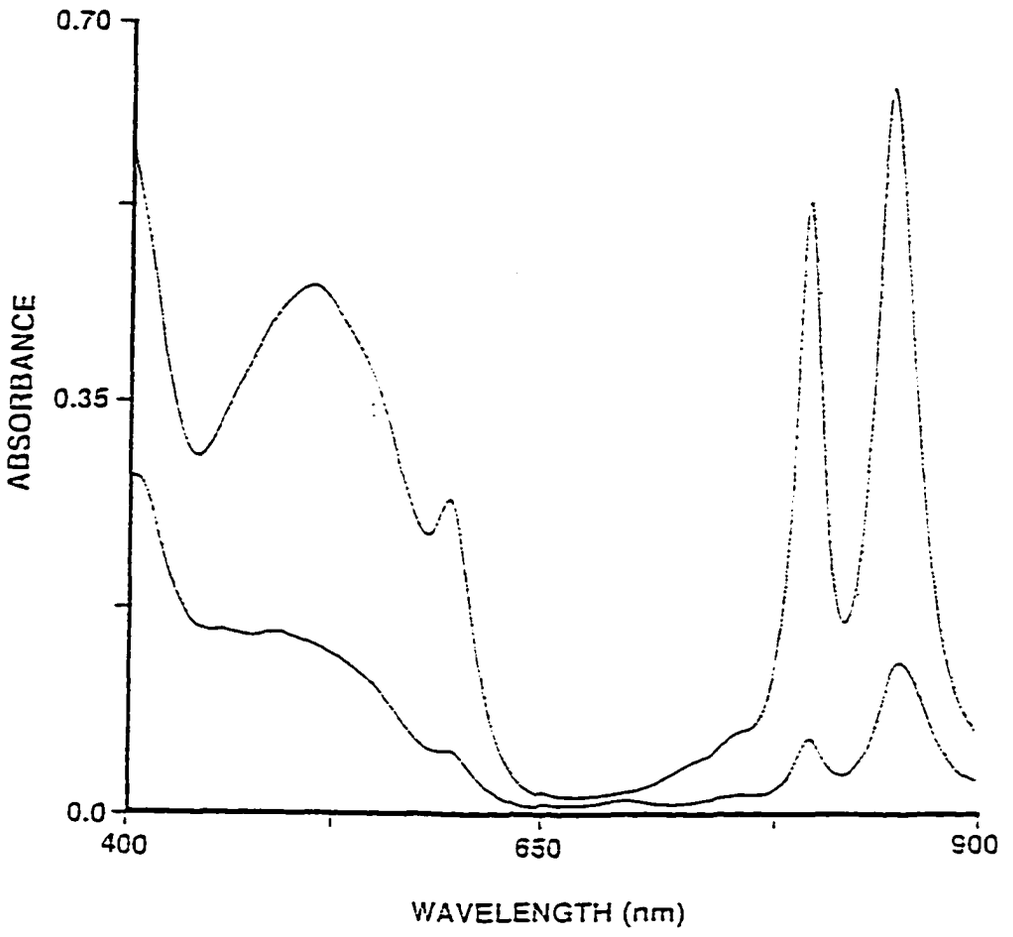


Figure 8. Absorbance spectra of PAS100 - upper scan and PAS100(pCAP150) - lower scan.

Table 4. Effects of overexpression of ferrochelatase on enzyme activities and porphyrin synthesis in *R. capsulatus* PAS100.

Strain	Ferrochelatase specific activity[a]	ALA synthase specific activity[b]	Bchl[c]	Porphyrin[d]
PAS100	208 ± 54	68 ± 10	5.3 ± 1.1	2.9
PAS100 (pCAP150)	735 ± 99	28 ± 5	1.2 ± 0.5	1.3

[a] Ferrochelatase specific activity is expressed as picomoles of deuteroheme formed per minute per milligram of protein (49). The results are reported as the average ± standard deviation.

[b] Aminolevulinate (ALA) synthase specific activity is expressed as nanomoles of aminolevulinate formed per hour per milligram of protein (30). The results are reported as the average ± standard deviation.

[c] Bacteriochlorophyll (Bchl) concentration is expressed as nanomoles per milligram of protein (39). The results are reported as the average ± standard deviation.

[d] Porphyrin concentration represents the concentration of total coproporphyrin and protoporphyrin expressed as nanomoles per milligram of protein.

The overall results suggested that the reduced accumulation of bacteriochlorophyll and porphyrins is probably a result of two factors: decreased synthesis of porphyrin due to lower aminolevulinate synthase activity and increased use of porphyrin since the higher ferrochelatase activity could convert protoporphyrin IX to heme much faster. These results suggested that heme feedback inhibits aminolevulinate synthase in *R. capsulatus*, thereby lowering the synthesis of porphyrins (coproporphyrin and protoporphyrin) and bacteriochlorophyll. This is the first *in vivo* evidence for such feedback inhibition of aminolevulinate synthase in *R. capsulatus*.

Effects of heme and protoporphyrin IX on ALA synthase activity in *R. capsulatus*

In order to confirm the *in vivo* evidence for feedback inhibition of aminolevulinate synthase by heme in *R. capsulatus*, the effect of heme on aminolevulinate synthase was tested *in vitro*. In addition, the effect of protoporphyrin IX, the direct precursor of heme, was also tested. Cultures of *R. capsulatus* PAS100 were grown to late exponential phase and cell extracts were prepared by sonication. Aminolevulinate synthase assays were performed with the addition of increasing concentrations of heme or protoporphyrin IX (1μM to 125μM) to separate reaction mixtures before incubation. The aminolevulinate synthase specific activity was determined for each reaction and the percent inhibition was calculated based on the aminolevulinate synthase specific activity in a control reaction containing no

heme or protoporphyrin IX. The results demonstrated strong inhibition of aminolevulinate synthase activity by heme *in vitro* (Fig. 9), which supports the *in vivo* evidence. Protoporphyrin IX, even in the highest concentration used, had no effect on the activity of aminolevulinate synthase.

As a control, the aminolevulinate synthase assay was performed in the presence of Tween 80, the solvent used in preparation of the heme solution. The Tween 80 solution was prepared exactly as when preparing a heme solution and the only ingredient it lacked was heme. Increasing amounts of Tween 80 solution corresponding to the heme amounts used in the inhibition assay were added to each enzyme reaction. The aminolevulinate synthase assay was performed the same way as described above. No inhibition of aminolevulinate synthase by Tween 80 was observed, supporting the fact that heme is the compound responsible for the inhibition.

Effects of extra *hemH* on the porphyrin accumulation in AJB530 at high and low oxygen tension

AJB530 is a Tn5 insertion mutant of the *R. capsulatus* strain PAS100. It accumulates porphyrins (coproporphyrin and protoporphyrin). It has been shown that around four-fold more porphyrins accumulate in AJB530 under low oxygen tension than under high oxygen tension (19). In order to determine if the feedback inhibition of aminolevulinate synthase by heme is related to the oxygen regulation of the tetrapyrrole pathway, the porphyrin accumulation in strains AJB530(pCAP150) and AJB530(pRK404) was determined.

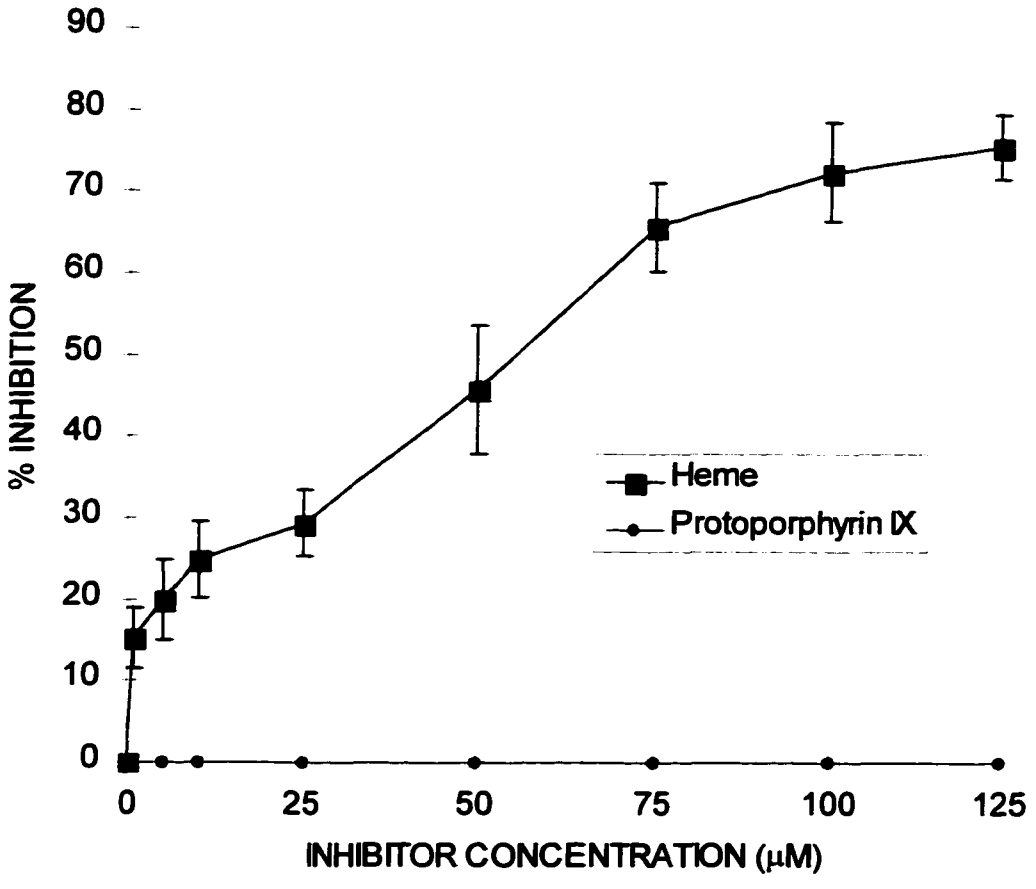


Figure 9. Heme and protoporphyrin IX inhibition of aminolevulinate synthase activity in *R. capsulatus*. Results are representative of six trials. The vertical bars indicate the standard error for each point.

AJB530(pCAP150) carries the *hemH* gene in the pRK404 insert. AJB530(pRK404) was used as a control strain because it carried only the vector pRK404. The cultures were grown in RCV medium under high and low oxygen tension. Porphyrins were extracted and their concentration determined. Porphyrin levels were 4.5 fold higher under low oxygen tension (0.68 nmoles/ml of culture) than under high oxygen tension (0.15 nmoles/ml of culture) in AJB530(pRK404) which is in agreement with previous measurements for AJB530 (19). Porphyrin levels were around three fold higher under low oxygen tension than under high oxygen tension in AJB530(pCAP150) (1.8 and 0.58 nmoles per ml of culture, respectively), which demonstrated that the presence of excess ferrochelatase does not influence the oxygen-mediated regulation of porphyrin accumulation in AJB530.

DISCUSSION

The versatile growth potential of the photosynthetic bacterium *R. capsulatus* and its ability to carry out the complex regulation of tetrapyrrole biosynthesis have made this organism an excellent system to study. The ability to grow under diverse environmental conditions and to accordingly regulate the amount of tetrapyrrole pathway end products with remarkable precision has been under extensive investigation.

Cloning, sequencing and mapping of the *R. capsulatus hemH* gene

Studying the regulation of tetrapyrrole biosynthesis in *R. capsulatus* has been the main goal of our laboratory. An essential step towards accomplishing this goal was the cloning, sequencing and characterization of all *hem* genes of the common tetrapyrrole biosynthetic pathway. So far four *R. capsulatus hem* genes have been cloned and sequenced: *hemA* (22), *hemB* (97), *hemC* (Canada and Biel, unpublished results) and *hemE* (95). Now, we report the cloning and sequencing of the fifth *R. capsulatus hem* gene, *hemH*, using an *E. coli hemH* deletion mutant. This leaves only three more *R. capsulatus hem* genes: *hemD*, *hemF* and *hemG* to be cloned and characterized which will be certainly completed in the near future.

The *R. capsulatus* ferrochelatase encoded by the *hemH* gene, with its deduced molecular mass of 46,000 Da, is similar to ferrochelatases of other

organisms (33,49,196). Interestingly, the *R. sphaeroides* ferrochelatase has a dramatically different molecular mass of 115,000 Da (44). The reason for that difference and its significance are not known, but it is tempting to speculate that although both *R. capsulatus* and *R. sphaeroides* are *Rhodobacter* species, they may have different regulatory mechanisms.

A physical map of the *R. capsulatus* genome created by Fonstein and Haselkorn (68) was used to determine the location of the *R. capsulatus hemH* gene, compared to the other *hem* genes mapped so far: *hemA*, *hemB* and *hemE*. All four *hem* genes are scattered throughout the chromosome and do not appear to form a cluster. Similar scattered distribution of *hem* genes was found in another Gram negative organism - *E. coli*. However, there are examples of clustered arrangement of *hem* genes such as in the Gram positive *B. subtilis* (81-82). Whether the clustered distribution of *hem* genes could be attributed to Gram positive organisms versus Gram negative organisms as well as its significance in the cellular regulatory mechanism of tetrapyrrole biosynthesis is not known.

Regulation of tetrapyrrole biosynthesis by oxygen and heme

In *R. sphaeroides*, it has been shown in an *in vitro* experiment that heme feedback inhibits aminolevulinate synthase, the first enzyme in the tetrapyrrole biosynthetic pathway (29). Based on these results, a model has been developed which suggests that in *R. sphaeroides* oxygen and heme participate in a single regulatory mechanism (164). According to that model,

oxygen prevents the conversion of protoporphyrin IX into magnesium protoporphyrin monomethyl ester. As a result, the accumulating protoporphyrin IX is converted into heme which feedback inhibits the aminolevulinate synthase causing a decrease in the carbon flow over the tetrapyrrole pathway.

Previous attempts to demonstrate *in vitro* inhibition of aminolevulinate synthase by heme in *R. capsulatus* have not been able to provide conclusive evidence for such inhibition. The results were variable, showing a maximum level of 30% inhibition of aminolevulinate synthase by heme (Biel, unpublished results). Because of that, another approach of testing for inhibition of aminolevulinate synthase by heme was followed. The levels of heme in *R. capsulatus* were increased *in vivo* by transferring additional copies of the *hemH* gene. A plasmid containing the *R. capsulatus hemH* gene, pCAP150, was transferred into *R. capsulatus* PAS100 by conjugation. A surprising finding was that the pigmentation of PAS100 and PAS100(pCAP150) cultures differed markedly. The wild type *R. capsulatus* strain PAS100 had a dark red color due to the synthesis of bacteriochlorophyll and carotenoids. On the other hand, the PAS100(pCAP150) culture had a light orange color which suggested that the increased ferrochelatase activity led to decrease of pigmentation in *R. capsulatus*. Extracts of both cultures were used to determine the absorbance spectra which showed a drastic decrease in the levels of both light-harvesting complexes as well as the carotenoid levels in

the *R. capsulatus* strain carrying additional copies of the *hemH* gene. In addition, measuring the levels of bacteriochlorophyll and porphyrins (coproporphyrin and protoporphyrin) from both cultures revealed that they were much lower in PAS100(pCAP150) than in PAS100 (Table 4). Ferrochelatase activity was also measured and, as expected, it was almost three fold higher in PAS100(pCAP150) due to the additional *hemH* copies. However, determining the aminolevulinate synthase activity in extracts of both cultures demonstrated lower aminolevulinate synthase activity in the *R. capsulatus* strain with extra *hemH* copies (Table 4). These results suggested, that heme feedback inhibits aminolevulinate synthase *in vivo*. Thus, it appears that the decreased level of porphyrins (coproporphyrin, protoporphyrin and bacteriochlorophyll) is due to both the preferential use of protoporphyrin IX by the increased ferrochelatase activity and the reduced synthesis of porphyrin down the tetrapyrrole pathway because of heme feedback inhibition of aminolevulinate synthase.

Repeating the *in vitro* experiment of aminolevulinate synthase inhibition by heme resulted initially in approximately 30% inhibition which was similar to previously obtained data (Biel, unpublished results). In order to improve the detection for heme inhibition, the aminolevulinate synthase assay was modified by including the appropriate concentrations of heme in the blank of each reaction mixture. The presence of heme in the blanks increased the absorbance. The results demonstrated a maximum level of approximately

70% inhibition of aminolevulinate synthase by heme which was reproduced in numerous experiments. Thus, we had a strong and conclusive evidence confirming the feedback inhibition of aminolevulinate synthase by heme in *R. capsulatus* both *in vivo* and *in vitro*.

Since our data confirmed the finding for *in vitro* inhibition of aminolevulinate synthase by heme in *R. sphaeroides*, we decided to determine if the model which connects oxygen and heme in a single regulatory mechanism in *R. sphaeroides* is true for *R. capsulatus*. The *R. capsulatus* Tn5 insertion mutant AJB530 is known to be regulated by oxygen (20). Therefore, it was used to create a strain, AJB530(pCAP150), containing additional copies of the *R. capsulatus hemH* gene introduced by pCAP150. This strain would be regulated by both oxygen and heme which would facilitate the study of whether both regulatory mechanisms are connected. Growing AJB530(pRK404) and AJB530(pCAP150) under high and low oxygen tension did not result in any changes in the usual porphyrin accumulation under both conditions. This demonstrated that in *R. capsulatus* regulation of tetrapyrrole biosynthesis by heme is separate from regulation by oxygen.

These results are also in agreement with previous observations by Biel (20). It has been shown already that oxygen controls protoporphyrin accumulation (21). Growing *R. capsulatus* under high oxygen tension resulted in a block in the step of magnesium protoporphyrin monomethyl ester

formation from protoporphyrin IX. Consequently, another step in the common tetrapyrrole pathway must be inhibited by oxygen to prevent accumulation of protoporphyrin. The initial hypothesis was that if oxygen is involved in inhibition of aminolevulinate synthesis, addition of exogenous aminolevulinate would overcome that inhibition and protoporphyrin would accumulate even under high oxygen tension. However, the presence of exogenous aminolevulinate did not affect the oxygen-mediated regulation of protoporphyrin accumulation which was still inhibited in AJB456, a *R. capsulatus bchH* mutant strain which has a block in the conversion of protoporphyrin to Mg-protoporphyrin monomethyl ester (20, 93). On the other hand, addition of exogenous porphobilinogen resulted in accumulation of protoporphyrin under both high and low oxygen growth conditions when AJB530, which accumulates and excretes coproporphyrin, was tested (20). This demonstrated that the oxygen-mediated control of protoporphyrin accumulation was disrupted by excess levels of porphobilinogen.

The attention was therefore directed towards studying the metabolism of porphobilinogen and its involvement in the oxygen-mediated control of tetrapyrrole biosynthesis. So far, using dot blot analysis of *R. capsulatus hemB* mRNA and *hemB-cat* fusion experiments, it was shown that there is no transcriptional regulation of the *R. capsulatus hemB* gene in response to high and low oxygen tension (97). Moreover, enzyme assay studies have clearly demonstrated that when the *R. capsulatus* cells were grown, cell extracts

made and enzyme activity determined under strictly aerobic or anaerobic conditions, the porphobilinogen synthase activity was not regulated by oxygen (Kanazireva and Biel, unpublished results).

It is possible that oxygen may influence the degradation of porphobilinogen. Preliminary results have demonstrated a two-fold variation in the ratio of porphobilinogen used to uroporphyrinogen formed in response to oxygen (Canada and Biel, unpublished results). However, it is not yet known what is the significance of that observation in the big picture of oxygen-mediated regulation of tetrapyrrole biosynthesis in *R. capsulatus*. Further investigation is required to establish exactly how the levels of porphobilinogen influence that regulation.

Regulation of tetrapyrrole biosynthesis by *c*-type cytochromes

Another factor implicated in the regulation of tetrapyrrole biosynthesis is *c*-type cytochromes. The *Rhodobacter capsulatus* Tn5 insertion mutant AJB530 is not capable of synthesizing *c*-type cytochromes (18). Consequently, this mutant strain accumulates about half of the amount of bacteriochlorophyll, compared to the wild type *R. capsulatus* strain PAS100. In addition, AJB530 accumulates up to 24-fold more coproporphyrin and protoporphyrin. Since AJB530 accumulates much more of these porphyrin intermediates than *R. capsulatus bchH* mutants, which are incapable of converting protoporphyrin into Mg-protoporphyrin monomethyl ester, it was proposed that *R. capsulatus* could increase the carbon flow over the common

portion of the tetrapyrrole pathway in order to synthesize more *c*-type cytochromes. However, the total porphyrin level which is a sum of the level of coproporphyrin, protoporphyrin and bacteriochlorophyll, was essentially the same in PAS100 and AJB530 (93). This suggested that AJB530 does not have higher carbon flow over the tetrapyrrole pathway. These results demonstrated that the tetrapyrrole pathway is not regulated by *c*-type cytochromes. However, we were left with the unexplained observation that *bchD* and *bchH* mutants have greatly reduced carbon flow over the tetrapyrrole pathway. Total porphyrin levels in both mutants were less than 5% of the total porphyrin level detected in AJB530 and PAS100.

The reduction in carbon flow over the tetrapyrrole pathway in *R. capsulatus* can be explained by feedback inhibition of aminolevulinate synthase. Our hypothesis was that there were two possible candidates to feedback inhibit aminolevulinate synthase: heme and protoporphyrin IX. As discussed previously, heme was shown to feedback inhibit aminolevulinate synthase both *in vivo* and *in vitro*. Previous studies in our laboratory had raised the possibility that protoporphyrin IX could be involved in feedback inhibition of aminolevulinate synthase (93). However, it was demonstrated in this study that protoporphyrin IX did not feedback inhibit aminolevulinate synthase in *R. capsulatus*.

In conclusion, the *R. capsulatus hemH* gene was cloned using complementation of an *E. coli hemH* deletion mutant and sequenced.

Mapping of the gene to the *R. capsulatus* chromosome showed no proximity to the other *hem* genes located so far, thus eliminating possibilities for possible clustered arrangement of the *hem* genes. Feedback inhibition of aminolevulinate synthase by heme was demonstrated both *in vivo* and *in vitro*. This is the first evidence for feedback inhibition of aminolevulinate synthase by heme in *R. capsulatus*. It was demonstrated that heme and oxygen do not participate in a single mechanism to regulate tetrapyrrole biosynthesis.

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VITA

Ekaterina V. Kanazireva was born on December 7, 1966, in Sofia, Bulgaria. She graduated from 19th High School with extended study of the German language, Sofia, Bulgaria, in May, 1984. Ekaterina received the degree of Master of Science in Microbiology and Molecular Biology from Sofia University, Sofia, Bulgaria, in May, 1990. She was employed as a Research Associate at the Institute of Molecular Biology - Bulgarian Academy of Sciences, Sofia, Bulgaria from September, 1990 to September, 1991. She was then transferred to Pharmagen Ltd, Sofia, Bulgaria, where she was employed as a Research Associate from September, 1991 to December, 1992. In January of 1993, Ekaterina entered the doctoral program in the Department of Microbiology at Louisiana State University, Baton Rouge under the direction of Dr. Alan J. Biel as her graduate advisor. Ekaterina is currently a doctoral candidate in the Department of Microbiology at Louisiana State University. In August of 1997 her doctor of philosophy degree will be granted at the summer commencement.

DOCTORAL EXAMINATION AND DISSERTATION REPORT

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Major Field: Microbiology

Title of Dissertation: Cloning, Sequencing and Regulation of the *Rhodobacter capsulatus hemH* Gene

Approved:

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

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Date of Examination:

6/23/97